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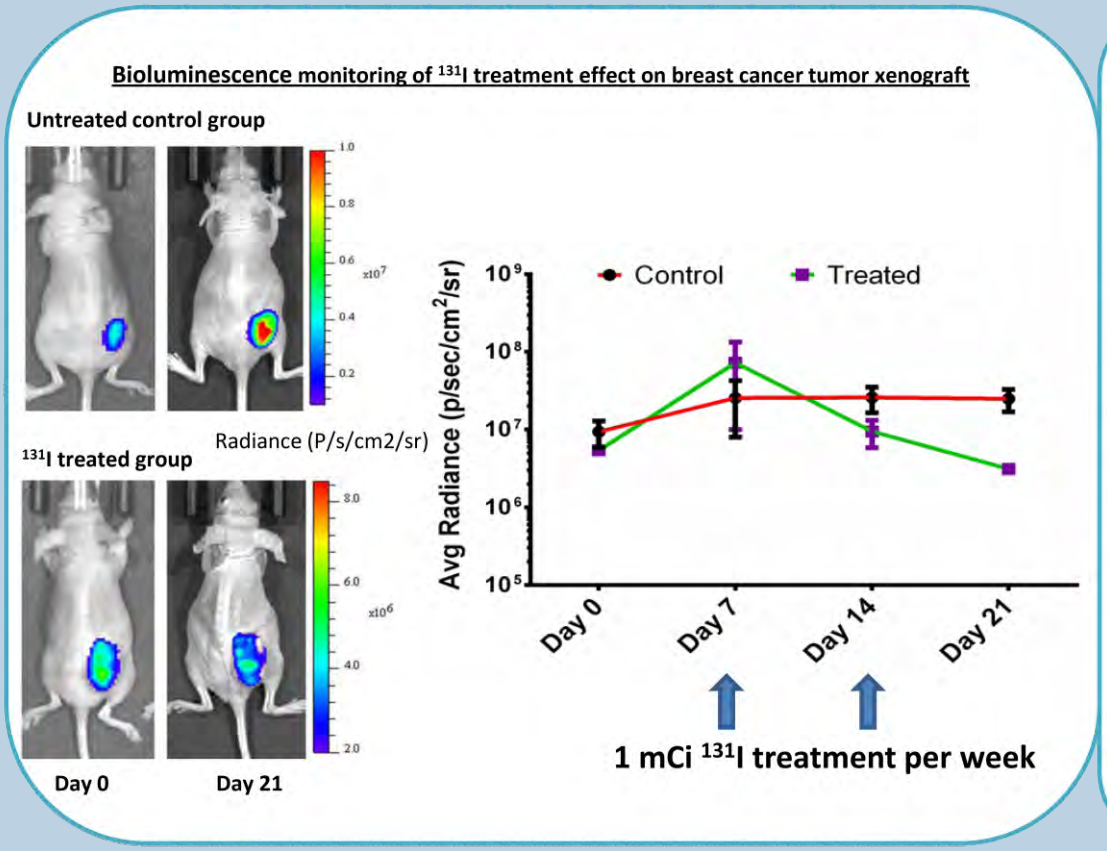


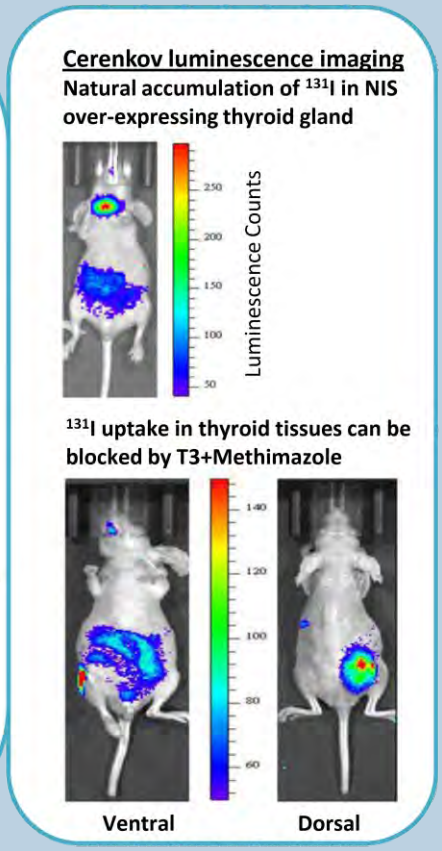


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# Biomedical Research Journal

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# Right Patient, Right Diagnosis, Right Treatment!

**Dhananjaya Saranath and Aparna Khanna**

The 2015 Nobel Prize in Medicine for drugs to fight malaria and other tropical diseases, and in Chemistry for fundamental contributions towards understanding DNA repair and maintaining of genomic integrity in cells, highlights the interdisciplinary approach for maximizing benefits of contemporary science to mankind. The Nobel Prize in Medicine was awarded to William Campbell Ph.D., born in Ireland and migrated to US; Satoshi Omura, Ph.D., from Japan, and Youyou Tu, the first Chinese Nobel laureate. The Nobel laureates – Dr. Campbell and Dr. Omura were cited for their discovery of Avermecitin, derivatives of the drug responsible for decreasing incidence of river blindness and lymphatic filariasis affecting millions in Asia and Africa. Ms. Tu's intensive efforts led to the active compound from the herbal Chinese sweet wormwood plant, giving us the antimalarial drug artemisinin, currently the first line drug for malaria affecting 50% of global population. The Chemistry Nobel acknowledged three scientists for their research in DNA repair, for their intensive work on mapping the process at a molecular level and providing insights into cell functioning and maintenance of genomic stability. The Nobel laureates were Dr. Thomas Lindahl, Ph.D., Francis Crick Institute, London, for his discoveries in base excision repair; Dr. Paul Modrich, Ph.D., Howard Hughes Medical Institute, and Duke University School of Medicine, North Carolina, USA, for the mismatch repair pathway; and Dr. Aziz Sancar, M.D., Ph.D., at the University of North Carolina, USA, for nucleotide excision pathway. The understanding of DNA repair mechanisms in the cells is a breakthrough in understanding how cancer develops and furthers treatment of cancer and also several diseases, much needed for better health management.

Despite the tremendous advances in technology, particularly biotechnology, information technology and imaging technology, cancer development,

progression, response to treatment, and recurrence is still an enigma with several glaring lacunae. It was in 1971, Richard M Nixon, 37th President of US, signed a bill, 'The National Cancer Act', to create new research infrastructure with enormous resources devoted to fighting cancer, and the act was known as 'Nixons war on cancer'. By 2005, NCI spent USD 165 billion, and the outcome was better understanding, and applications of the generated data resulted in reduced mortality from cancer in both men and women. The outcome of genome based screening in Cervical Cancer, Breast Cancer, Colon Cancer and other cancers was early detection, better prognosis and better survival. Genome wide association studies gave us 'Predictive markers', and the whole genome expression studies through transcriptomics, proteomics, metabolomics gave us 'Prognostic Biomarkers' and identified 'Drug Targets' with cutting edge technology. The 'Next Generation Sequencing' with massive parallel sequencing over the past several years, is anticipated to be an invaluable part of clinical medicine.

With the current 1.2 billion population in India, preventive medicine often takes a back seat, although the focus of health management in developed and developing world should be 'Prevention' in monogeneic and multigeneic complex diseases including 'Cancer'. Despite oral cancer ranked as the number one cancer in Indian males and fourth most common cancer in females, contributing 26% of the global oral cancer burden, preventive measures are slow in implementation. Tobacco has been unequivocally established as a high risk factor in oral and lung cancers; however, the role of areca nut is not commonly acknowledged as an important risk factor for oral cancer. In the current issue Dr. Prakash Gupta and Cecily Ray, Healis Sekhsaria Institute for Public Health, Mumbai, in their article on 'Areca nut use and cancer in India', give us a comprehensive review of the evidence for carcinogenicity of areca nut, detail epidemiological and animal studies, and reveal the mechanistic evidence highlighting the causal biochemical and molecular mechanisms of oral submucous fibrosis – a premalignant condition and oral cancer in areca nut chewers. The review emphasizes the necessity for awareness programs for areca nut hazards and control policies on areca nut per se and areca nut products.

Inherent in all health management

programs is the understanding of the mechanisms of diseases using state of the art technology. The concepts of 'Systems Biology' and 'Omics' was knowledge based and advanced applications in clinical medicine. Dr. Rukmini Govekar, Advanced Centre for Treatment, Research and Cancer, Navi Mumbai has lucidly highlighted proteomic technologies and strategies in her article 'Identification of Therapeutic Technologies and Strategies are the Key to Success'. The 'Omics' research has immensely contributed to

'Targeted therapy' in cancer, and is a rapidly resulting in pathology specific therapeutic molecules relevant to an expanding list of tumor types. Thus, the targeted therapy is useful not only in the initially indicated cancer, but is useful in several cancers with identical or similar molecular pathology. The therapy is tailored to the individual patient, with 'Companion Diagnostics' giving a helping hand in the informed decision for the patient. Off label use of targeted therapies will be indicated as confirmed data such as BRCA1/BRCA2 gene mutations as predictive diagnostic test for breast cancer, and hence use of targeted therapy with the specific molecular pathology, is useful in Pancreatic cancers and Non-Small Cell Lung Cancer patients. The recent immune checkpoint blockade therapies, Anti-PDL1, may be used effectively in advanced melanoma or metastatic bladder cancer with impressive clinical responses. A massive effort for high incidence and rare cancers is the NCI-Molecular Analysis for Therapy Choice (NCI-MATCH) Trial, world's largest ever molecular oncology trial, screening 3000 patients for comprehensive molecular pathology, and treated with targeted therapy. Biopsy specimens from tumors will be analyzed for more than 4,000 different variants across 143 genes, regardless of tumor origin. Dr. Pratibha Kadam Amare, Tata Memorial Hospital, Mumbai, gives a snap shot of molecular pathology and targeted therapy in her review on 'Genetic markers and evolution of targeted therapy in cancer'. The author elaborates the cell surface antigens and tyrosine kinase targets identified as pathognomonic in several common cancers, and development of effective inhibitor molecules as small molecules or antibodies for therapy, a must read for our oncologists and basic scientists.

The re-emergence of 'Gene Therapy' recently is obvious. The field has matured, gathering steam and is anticipated to join

mainstream therapy in several monogenic diseases as also complex diseases including cancer, after several years of taking a backseat in both the research realm and clinical applications. The falling precipice in gene therapy truly began in 1999 when 18 year old Jesse Gelsinger died from multiple organ failure four days after treatment for ornithine trans-carboxylase deficiency, the death triggered by severe response to adenoviral carrier. The disenchantment with gene therapy continued when several children developed leukemia like condition after treatment for X-linked severe combined immunodeficiency disease. The current scenario is quite different with several companies conducting clinical trials with gene based therapy using AAV vector, oncolytic viruses, liposomes coated with Polyethylene Glycol, siRNA, etc. And the clinical trials using gene therapy in varied diseases – Parkinson disease, the drug used is Nerologix (Biotech Company); A successful correction of inherited form of blindness has been launched by Spark Therapeutics; Inhalation of normal Cystic Fibrosis CFTR gene copies for improvement in lung function for Cystic fibrosis patients. Gene therapy with siRNA to degrade particular RNA sequences is under investigation for Huntington's disease; and lentivirus based gene transfer to improve efficiency of gene transfer in metachromatic leukodystrophy with the pathology being mutated arylsulfatase A enzyme. The lentivirus vector avoids activation of cancer causing genes by loading the vector with self-inactivating promoter sequences that exclusively induce expression of the therapeutic gene. The advances in gene delivery systems will relegate complex diseases such as cancers to a manageable disease without severe disability, disfigurement and death. Dr. Abhijit De and Shruti Dutta in their article 'Gene therapy for sodium iodide symporter in non-thyroidal cancers' focus on potential suicide genes in cancer supporting diagnosis through imaging of the cancer and therapeutic applications, with emphasis on sodium iodide symporter. The authors emphasize the effectiveness of sodium iodide symporter in non-thyroidal cancers, particularly hormone receptor negative patients.

The technology today facilitates a 'Systems Biology' approach for understanding the role of 'microbiome' at different sites of our body, with emergence of functional metagenomics, to enable a

holistic picture of the beneficial normal microbes. Subtle imbalances in our microbial population can cause diseases, influences our response to drugs, and restore balance between microbial normalcy and pathogenesis, leading to effective cures. Global parameters of microbial communities provide valuable information regarding human health status and disease predisposition. Downstream analyses of the functional interaction between host and the microbiome deciphering the metagenome i.e. the human microbiome and its collective genes, may lead to mechanistic insights into the interaction, with new opportunities for next generation diagnosis, prognosis and treatment of various acute, chronic, localized, systemic, simple and complex diseases. *In vitro* cultivation of the microbiota earlier formed the cornerstone in microbiology, though not possible in densely populated microbial communities. DNA based analyses expands the horizons generating enormous new data sets mined for information. The Human Microbiome Project (by NIH), generated 2.3 terabyte 16S rRNA metagenomics dataset of over 35 billion reads from 690 samples of 300 subjects across 15 body sites. The microbiome and core metagenomics occupies a specific human niche, and varies both by anatomical site and substantial interpersonal variation, with a causal link established between microbiome variation and significant pathology. Dietary changes rapidly cause substantial metagenomic changes. Dr. Abhay Kumar Pandey, Banaras Hindu University, Varanasi, and colleagues succinctly highlight the important role of the microbiome in disease and health in the review 'Microbiota in immune pathogenesis and the prospects for Pre and Probiotic Dietetics in psoriasis'. Psoriasis is a chronic idiopathic, inflammatory dermatologic condition, with the cutaneous microbiome a critical target. The pathogenesis is advanced by genetic predisposition, and a change in the microbiota is observed, with increased ratio of Firmicutes to Actinobacteria. The alterations in commensal microbiome, basis for the changes in disease, and the mechanisms of colonization and host homeostasis is restored, is crucial for manipulating the host microbiota. The authors elaborate the role of dietary prebiotics and probiotics towards healthy host microbiome relationship via dietary probiotics and manipulation of the specific

site and general gut microbiota. It is feasible that the advances in technology facilitating better understanding of disease pathogenesis will prove fruitful and provide optimal benefits and the end of 'Right diagnosis, Right treatment, Right patient,' is in sight, making therapeutics robust and dynamic.

Review

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# Areca Nut Use and Cancer in India

**Prakash C. Gupta and Cecily S. Ray***

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Areca nut is widely used in India and the consumption has increased over the past two decades, with availability in new dry packaged forms (*pan masala, gutka, mawa*). Recent reports of increasing mouth cancer incidence have suggested an association with areca nut consumption. Here we have reviewed the evidence for carcinogenicity of areca nut, including epidemiological studies, several animal studies and mechanistic evidence. Studies primarily from India, providing odds ratios (ORs) or relative risks for precancers or cancer with use of areca nut without inclusion of tobacco is the focus of the review. Six case-control studies on oral submucous fibrosis (OSF) had significantly elevated ORs for use of areca nut in various forms. Six case-control studies on head and neck cancers, primarily oral cancer reported elevated ORs for chewing of betel quid without tobacco. Eight case control studies on oral cancer have reported elevated and significant ORs for betel quid with tobacco. A significant risk in oral cancer was noted in *gutka* users. Animal studies confirmed correlation between development of precancers or cancers and exposure to areca nut or *pan masala* without tobacco. Mechanistic evidence shows a role for areca nut alkaloids, polyphenols and copper in promoting carcinogenesis. Our review emphasizes control policies on areca nut products and appropriate mass communication programs for awareness of hazards of areca nut with emphasis on areca nut *per se*.

## INTRODUCTION

The areca nut, fruit of the oriental palm (*Areca catechu*), also called 'betel' nut in English, *supar*i in Hindi, *adike* or *betta* in Kannada, *adakka* in Malayalam, and *pakku* in Tamil, is commonly used in India (FRLHT.org, 2015) and needs no introduction. It is used in traditional quids (*beeda*) wrapped in betel leaves (*Piper betle*) or as tobacco and areca nut mixtures. Areca nut is also used *per se* and available in specialised shops and by roadside vendors, in sachets, as *pan masala* and *gutka*. A product containing areca nut chips, slaked lime and tobacco, popularised in Gujarat, is called *mawa* (Gupta, 1998), also sold in Maharashtra as *kharra* (Hazare *et al.,* 1998). *Mainpuri* tobacco containing similar ingredients is

**Key words**: Areca nut, Prevalence, India, Oral submucous fibrosis, Leukoplakia, Neoplasms, Case control studies, Laboratory studies, Oxidative stress, DNA damage.
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consumed in Uttar Pradesh, since the 1960s (Wahi, 1968). These commercial developments resulted in doubling areca nut consumption in India during 1991 to 2010 from 2.5 to 5.2 lakh tons, with about 5% increase each year (Kammardi *et al.,*2012).

A recent report of the National Cancer Registry Programme (ICMR) showed an increasing incidence of cancer of the buccal mucosa ('mouth cancer') for six to ten years up to 2009 or 2010 in five of the nine population-based cancer registries (NCDIR-NCRP, 2013), reiterated by a similar trend in a single registry (Gupta *et al.,* 2014). The cancer registries located in Bhopal, Mumbai, Delhi, Dibrugarh and Ahmedabad rural and urban, in the states or territory of Madhya Pradesh, Maharashtra, Delhi Union Territory, Assam and Gujarat, respectively have high prevalence of high areca nut use (IIPS & MOHFW, 2010). In addition to the increased incidence, patients of oral cancer are younger than 35 years of age since the mid-1990s as compared to the mid-1980s (Gupta, 1999).

Betel quid has been linked with head and neck cancers including oral cancers since the last century, although at that time tobacco and lime in the betel quid were viewed as the likely causes of the associated cancer (IARC, 2004; Orr, 1933). In the past decade, oral cancer has been diagnosed with increasing frequency in young users (< 35 years) of packaged areca nut products, bringing the potential carcinogenicity of areca nut into focus (Chaudhry, 1999; Gupta, 1999). An extensive review and evaluation of evidence was undertaken on areca nut and betel quid by the International Agency for Research on Cancer (IARC) reported in 2004. The evidence for carcinogenicity of areca nut, primarily from India and South Asia was from use of betel quid with tobacco. Relatively few epidemiological studies on precancers or cancer, in the past twenty years reported on cancer risks associated with use of betel quid without tobacco or use of industrially manufactured areca nut products. Nevertheless these few studies, along with laboratory evidence, made it possible for the monograph to conclude that areca nut by itself is carcinogenic to humans. The final evaluation by IARC concluded that betel quid without tobacco causes cancer of the oral cavity, and betel quid with tobacco causes cancer in the oral cavity, pharynx and esophagus; and emphasized that areca nut is carcinogenic to humans

(IARC, 2004). In the most recent monograph (Vol. 100E) several additional studies were reviewed and the evidence confirmed carcinogenicity of areca nut in humans and animals. However, the message has apparently not reached the masses, perhaps ignored or discounted, in view of the overwhelming evidence of carcinogenicity of tobacco.

Thus, in view of increasing consumption of areca nut products in India and reports of increasing oral cancer incidence over the past ten years, a review of currently available evidence of the carcinogenicity of areca nut was undertaken. An initial literature survey of the use of areca nut products in India, followed by epidemiological and laboratory evidence for the role of areca nut in causing oral cancer and other head and neck precancers, and an outline of the mechanisms of cancer causation are reviewed.

## MATERIALS AND METHODS

Literature on carcinogenicity of areca nut and its products as used in India (areca nut, betel quid or *paan*, *gutka*, *pan masala, mawa*) was surveyed. Since use of areca nut without tobacco has been rare, earlier epidemiological studies have generally not reported separate risks for areca nut. However, since tobacco is widely recognised as carcinogenic, and areca nut has not been associated with cancer we focused on case-control studies that reported ORs for use of areca nut without tobacco. Epidemiological studies reporting on oral precancers or oral and pharyngeal cancers are included. The evaluation monographs of the IARC, volumes 85 (2004) entitled, "Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines" (2004), and 100E (2009) on *"*Betel quid and areca nut*"* were used as the basic resources, along with internet searches in Pubmed for case control studies, cohort studies, animal experiments and mechanistic studies. The more recent studies are emphasized, with a few highly informative earlier studies included. Certain studies on oral submucous fibrosis (OSF) not reviewed in the IARC Monographs are emphasized (Bathi *et al*. 2009; Mehrotra *et al*., 2013). Research conducted in India is prioritized, and additional studies in other parts of the world cited to provide evidence of areca nut as an important carcinogen globally are included. In addition, basic prevalence data on use of areca nut products were obtained from the Global Adult Tobacco

Survey for India (GATS) (International Institute for Population Sciences and Ministry of Health and Family Welfare, Government of India, 2010).

## RESULTS

### Prevalence of Areca nut Use in India

The report of the GATS for India showed betel quid with tobacco was used by 7.5% men and 4.9% women, and mixtures of areca nut and tobacco, without betel leaf (*gutka* and *mawa*) used by 13.1% men and 2.9% women. The report did not provide data for use of areca nut without tobacco (Table 1). The data showed that use of pre-packaged imperishable forms of areca nut have superseded the popularity of betel quid.

In rural areas, the prevalence of betel quid with tobacco was higher in urban areas (6.8% rural vs. 4.8% urban), prevalence of *gutka* and similar products in rural areas was higher than in urban areas (8.6% rural vs. 7.1% urban). The regions with high prevalence of use of areca nut products in India were the

**Table 1:** Prevalence of use of products containing areca nut and tobacco among persons ≥ 15 years in India, from the GATS (International Institute for Population Sciences and Ministry of Health and Family Welfare, Government of India, 2010).

| Substances used | *All current users*<br>N<br>% | Males<br>N<br>% | Females<br>N<br>% |
|---|---|---|---|
| *Gutka* & other areca nut mixtures with tobacco and lime | *65,072,000*<br>8.2 | 13.1 | 2.9 |
| | Range: 2.8–12.1<br>North to Central | Range: 4.9–18.4<br>North to Central | Range: 0.2–5.0<br>North to Central |
| Betel quid with tobacco: | *49,672,000*<br>6.2 | 7.5 | 4.9 |
| | Range: 0.7–17.2<br>North to Northeast | Range: 1.1–18.9<br>North to Northeast | Range:0.3–15.6<br>North to Northeast |
| *Pan masala* or betel quid without tobacco; and/ or use of nasal snuff | *35,106,000*<br>4.4 | 3.5 | 5.4 |
| | Range: 0.8–10.9<br>North to East | Range: 1.0–10.5<br>North to Northeast | Range: 2.3–15.0<br>West to East |

**Note:** Total prevalence could not be calculated from these values as the categories are not mutually exclusive.
**Key to regions:**
**North-East:** Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya and Assam
**East:** West Bengal, Jharkhand, Odisha and Bihar;
**Central:** Rajasthan, Uttar Pradesh, Chhattisgarh and Madhya Pradesh;
**North:** Haryana and northwards including Jammu & Kashmir, Himachal Pradesh, Punjab, Chandigarh, Uttarakhand and Delhi.

Northeast (Sikkim, Arunachal Pradesh, Nagaland, Manipur, Mizoram, Tripura, Meghalaya and Assam), the East (West Bengal, Jharkhand, Odisha and Bihar) and the Central region (Rajasthan, Uttar Pradesh, Chhattisgarh and Madhya Pradesh). Low prevalence was found in the North (Haryana and northwards including Jammu & Kashmir, Himachal Pradesh, Punjab, Chandigarh, Uttarkhand and Delhi). In particular, prevalence of betel quid with tobacco was high in the Northeast (17.2%) and East (9.7%), and lowest in the North (5.5%). On the other hand, prevalence of *gutka* and similar mixtures was high in the Central states (12.1%). Among men, *gutka* use was concentrated among the 15 to 44 year age group, whereas women users tended to be older. Betel quid with tobacco was used mainly among the 45–65 year age groups in both men and women (International Institute for Population Sciences and Ministry of Health and Family Welfare, Government of India, 2010).

Occasional consumption of areca nut without betel leaf, lime and condiments has been a norm and a common culturally accepted practice in India (Reddy and Gupta, 2004). Areca nut consumption without tobacco and by itself has been occasionally reported as practiced by a small fraction of the population before the 1980s (Mehta *et al.,* 1972). In the last 10–15 years areca nut habits have been observed in children (Chaturvedi *et al.,* 2002; Khandelwal *et al.,* 2012).

**Evidence of Carcinogenicity in Humans**

In India, a quid containing areca nut is chewed and kept next to the cheek (buccal) mucosa, for hours including overnight. Blanching often appears at the site as an early sign of OSF and squamous cell carcinoma may develop. Various case-control studies on precancers and cancers associated with areca nut use are summarised in the following section.

***Oral Precancers***

Six case control studies on OSF, five from India in the states of Bihar, Gujarat, Kerala, Karnataka and Uttar Pradesh (Ahmad *et al.,* 2006; Bathi *et al.,* 2009; Jacob *et al*., 2004; Mehrotra *et al.,* 2013; Sinor *et al.,* 1990) and one from Sindh in Pakistan (Maher *et al.,* 1994), showed significantly elevated ORs for OSF associated with areca nut use without tobacco in various forms (Table 2). ORs for OSF for betel quid without tobacco (BQ) ranged from 1.3, the lowest, which

**Table 2.** Oral submucous fibrosis and use of areca nut (AN) in various forms, in case control studies conducted India and Pakistan (both men and women).

| Location | Chewing status | OSF Cases | Controls | OR (CI) OSF | Study type. Matching, Adjustments, Stratifications | References Notes |
|---|---|---|---|---|---|---|
| Bhavnagar, Gujarat, India | Non Chewer (currently)[a] | 1 | 39 | 1.0 (ref.) | Dental clinic based. Age ≥15 yrs. Matched on age, sex, religion, and occupation. | (Sinor *et al.,* 1990) Significant dose response for frequency & duration |
| | BQ or AN (no tobacco) | 4 | 2 | 78.0* | | |
| Karachi, Pakistan | None (includes ex-chewers > 6 m) | 2 | 82 | 1 (ref.) | Dental clinic based. Matched on sex and age. | (Maher *et al,* 1994 ) Significant dose response for frequency & duration |
| | BQ (no tobacco) | 7 | 9 | 32 (6–177)** | | |
| | AN alone | 64 | 17 | 154 (34–693)** | | |
| Kerala (rural), India | No chewing (currently) | 9 | 31884 | 1.0 (ref.) | Population based. Age >35 yrs Adjusted for age, sex, education, smoking and alcohol drinking. | (Jacob *et al.,* 2004) Significant dose response for frequency |
| | BQ (no tobacco) | 15 | 1100 | 47.2 (20.2–110.4) | | |
| | Areca nut only | 0 | 12 | - | | |
| Patna, Bihar, India | No areca nut product use | 5 | 108 | 1.0 (ref.) | Dental clinic based. Matched on age, sex, religion & socio-economic status. ORs calculated by review authors | (Ahmad *et al.,* 2006) |
| | BQ not specified | 25 | 13 | 41.5 (13.5–127.2) | | |
| | AN alone | 8 | 1 | 172.8 (18.0–1662.6) | | |
| | *Pan masala* | 32 | 5 | 138.2 (37.6–507.7) | | |
| Dharwad, Karnataka, India | No habit (currently) | 1 | 119 | 1.0 (ref.) | Hospital based. Matched on age, sex, and socio-economic status. | (Bathi *et al.,*2009) Dose response calculation included diverse product users, not informative |
| | *Pan masala*/BQ/areca nut | 2 | 4 | 59.5 (3.1–2154) | | |
| | BQ (no tobacco) | 23 | 43 | 63.6 (8.7–1304) Confidence intervals recalculated by review authors | | |
| Lucknow (urban, rural), Uttar Pradesh, India | Non users (currently) | NA | NA | (ref.) | Subjects from urban and rural health camps Matched on age, and socio-econ. status. Males: (89.1% cases); (82% controls). | (Mehrotra, *et al.,* 2013) Dose response seen for frequency per day for each product |
| | Tobaccoless products | | | | | |
| | BQ | | | 1.3 (0.95–2.7) | | |
| | *Pan masala* | | | 3.0 (1.2–7.4) | | |
| | Both | | | 6.4 (4.3–9.5) | | |
| | Total visitors to camps | 448 | 2688 | | | |

CI= Confidence intervals; AN=areca nut; BQ=Betel quid without tobacco; BQT= Betel quid with tobacco.

[a]Occasional chewer of AN

*$P < 0.01$, **$P < 0.0001$

did not reach significance (Mehrotra *et al.,* 2013) to 78.0 (Sinor *et al.,* 1990). In contrast, ORs for BQ with tobacco ranged from 7.9 (Mehrotra *et al.,* 2013) to 64 (Maher *et al.,* 1994). Use of areca nut alone reported in two studies had ORs of 154 (Maher *et al*., 1994) and 172 (Ahmad *et al*., 2006). ORs exclusively for tobacco-less *pan masala* use in two studies were 3.0 (Mehrotra *et al*., 2013) and 138.2 (Ahmad *et al*., 2006).

Ors tended to be higher for users of mixtures made with areca nut and tobacco but without betel leaf, such as *mawa* (106.4) (Sinor *et al.,* 1990), or *gutka* (from 10.8 to 1142) (Bathi *et al.,* 2009; Mehrotra, *et al.,* 2013). Additionally, a cross-sectional house-to-house study showed an OR for men with OSF as 75.6 for *mawa* chewing in 11,262 men in Bhavnagar District of Gujarat (Gupta *et al*., 1998).

Studies were not adjusted for smoking, with one exception that also studied leukoplakia (Jacob *et al*., 2004). However, the report of the earliest study stated the rate of smoking in cases and controls was similar and also that smoking did not appear play a role in the development of OSF (Sinor *et al*., 1993). Another study performed multiple logistic regression on smoking and OSF and reported negligible effect of smoking (Bathi *et al*., 2009). Few smokers were found among chewers in a study and they were excluded from calculation of ORs (Maher *et al.,* 1994). Mehrotra *et al* (2013) concluded that tobacco smoking did not affect risk of OSF, whereas alcohol consumption increased the risk in chewers of tobacco-less betel quid or *pan masala* several fold.

A dose response was clearly seen for frequency per day of using areca nut preparations in four of the studies (Jacob *et al.,* 2004; Maher *et al.,* 1994; Mehrotra *et al.,* 2013; Sinor *et al.,* 1990). A clear dose response was also clearly seen for duration (Jacob *et al*., 2004; Maher *et al*., 1994; Sinor, *et al.*, 1990).

Two case control studies, one with betel quid and *pan masala* (Shah and Sharma, 1998) and the other with *pan masala, kharra,* tobacco-lime and betel quid in different combinations (Hazare *et al.,* 1998), reported significant increasing trends for frequency of use of areca nut containing substances per day ($p < 0.01$), although overall ORs for OSF was not reported. An increasing prevalence of OSF was observed between 2000 and 2004 with 77.8% of OSF patients using multiple areca nut products (Hazare *et al.,* 2007).

Table 3. Selected precancers and use of areca nut without tobacco in Kerala, India (Both men and women).

| Location | Chewing status | Cases | Controls | OR (CI) | Study type. Matching, Adjustments, & Stratifications | Reference |
|---|---|---|---|---|---|---|
| Kerala | | | | Leukoplakia: | Population based. | (Jacob *et al.,* 2004) |
| | Non chewers | 176 | 31884 | 1.00 (ref.) | Aged >35 yrs. | |
| | BQ | 27 | 1100 | 4.0 (2.7–6.1) | Adjusted for smoking & | |
| | AN only | 1 | 12 | 12.8 (1.6–101.2) | alcohol drinking. | |
| | | | | Erythroplakia: | | |
| | Non chewers | 8 | 31884 | 1.00 (ref.) | | |
| | BQ | 4 | 1100 | 12.5 (3.70–42.4) | | |

CI = Confidence intervals. BQ = Betel quid without tobacco; AN = Areca nut. Note: Use of lime may be inferred.

**Leukoplakia**

Betel quid chewing with or without tobacco has been associated with leukoplakia, a precancerous lesion, as reported in case series, case-control, cross-sectional and cohort studies (IARC, 2004). A case control study from Kerala (Jacob *et al.,* 2004), reported an OR of 4.0 for chewers of betel quid without tobacco, and OR of 12.8 (1.6–101.2) for chewers of areca nut by itself, that may include lime. Both the ORs were adjusted for smoking (Table 3). The trends for both frequency and duration were significant ($p < 0.0001$). The OR for chewers of BQ with tobacco was 10.0 (8.3–12.0) and that for tobacco only was 30.9 (13.7–69.7). The study also showed an OR of 12.5 (3.70–42.4) for erythroplakia, a rarer lesion.

***Oral Cancer and Other Head and Neck Cancers***

Eight case control studies on oral and other head and neck cancers, in India are summarised in Table 4, conducted in Madhya Pradesh (Dikshit and Kanhere, 2000), Maharashtra (Jussawala and Deshpande, 1971; Wasnik *et al.,* 1998), and southern Indian states of Kerala, Karnataka and Tamil Nadu (Balaram *et al.,* 2002; Mahapatra, 2015; Muwonge *et al.,* 2008; Nandakumar *et al.,* 1990; Znaor *et al.,* 2003), with two studies being multi-centric, and two studies in men only. Five of the studies adjusted for tobacco smoking, one also for oral dip products (smokeless tobacco) and four adjusted for alcohol.

Six of the studies showed elevated ORs for cancer and chewing of betel quid without tobacco. In the two smallest studies, the ORs were not significant (Dikshit and Kanhere, 2000; Nandakumar *et al.,* 1990). The study from Trivandrum, Kerala, reported an elevated and significant OR on chewing of areca nut

**Table 4.** Areca nut chewing practices ad risk of oral and other head and neck cancers in case control studies in India.

| Sex / Location/ | Cancers | Chewing status | Cases | Controls[#] | Odds ratio (CI) for cancer | Study type. Matching, Adjustments, & Stratifications | References Notes on trends |
|---|---|---|---|---|---|---|---|
| **BOTH MEN & WOMEN** | | | | | | | |
| Mumbai, Maharashtra | Oral cavity, pharynx, esophagus, and larynx: ICD 9 codes 140–148, 150, 161. | Non chewers | 129 | 1340 | 1.0 (ref.) | Population based. Matched on age, sex, and religion. Not adjusted. | (Jussawala and Deshpande, 1971) |
| | | BQ | 44 | 152 | 3.0 oral cavity* 1.0 (ref.) | | |
| | | Non chewers | 106 | 152 | 3.0 oropharynx* | | No analysis for trends. |
| | | BQ | 106 | 152 | | | |
| Bangalore, Karnataka | Oral cancer: ICD 9 sites for lip, tongue (excluding base of the tongue), alveolus, and mouth | Never chewers | 87 | 233 | 1.0 (ref.) | Hospital based. Matched on age, sex, and area of residence. | (Nandakumar *et al.,* 1990) |
| | | BQ | 24 | 45 | 1.7 (0.9–3.5) NS | Adjusted for smoking. | Significant trends for frequency and duration of chewing in general. |
| Nagpur, Maharashtra | Oro-pharyngeal cancers, ICD 9 codes not specified. | Non chewers | 33 | 185 | 1.0 (ref.) | Hospital based. Matched on age and sex. Univariate. | (Wasnik *et al.,* 1998) |
| | | Areca nut | 5 | 14 | 2.6 (0.9–7.7) NS | | Significant trends for frequency and duration of use. |
| | | BQ | 7 | 18 | 2.8 (1.1–7.4) | | |
| Trivandrum, Kerala | Oral cancer: ICD 10 codes C001–C009 | Never chewers | 80 | 915 | 1.0 (ref.) | Population based. Matched on age & sex. Adjusted for education, religion, smoking and alcohol drinking. | (Muwonge *et al.,* 2008). Significant trends for frequency and duration of use. |
| | | BQ | 13 | 44 | 3.5 (1.7–7.1) | | |
| Manipal, Karnataka | Oral cancer: ICD 10 codes Not specified | Supari : | | | | Hospital based. Unmatched. Adjusted for age, sex, social class, education level, diet, other tobacco types, dip products, and alcohol. | (Mahapatra, *et al.,* 2015) No analysis for trends. |
| | | No | 114 | 261 | 1.0 (ref) | | |
| | | Yes | 20 | 7 | 11.4 (3.4–38.2) | | |
| | | BQ (T): | | | | | |
| | | No | 110 | 257 | 1.0 (ref) | | |
| | | Yes | 24 | 17 | 6.4 (2.6–15.5) | | |
| | | Incorporation of tobacco not specified | | | | | |

Contd…

**Table 4.** Areca nut chewing practices ad risk of oral and other head and neck cancers in case control studies in India (Contd…)

| Sex / Location | Cancers | Chewing status | Cases | Controls[#] | Odds ratio (Adj.) & CI for oral cancer | Study type, Matching, Adjustments, & Stratifications | Reference |
|---|---|---|---|---|---|---|---|
| **MEN** | | | | | | | |
| Bangalore, Karnataka | Oral cancer: ICD 9 sites for lip, tongue (excluding base of the tongue), alveolus, and mouth | Never chewers<br>BQ | 68<br>15 | 89<br>15 | 1.0 (ref.)<br>1.5 (0.6–3.8)<br>NS | Hospital based. Matched on age, sex, and area of residence. Adjusted for smoking. | (Nandakumar *et al.,* 1990)<br>Significant trends for frequency and duration of chewing in general. |
| Bhopal, Madhya Pradesh | Oral cavity & orophayrynx: ICD 9 codes 140, 141, 143–145, 146–149 | Non chewers<br>BQ | 28<br>4 | 140<br>12 | 1.0 (ref.)<br>1.7 (0.9–3.3NS | Population based. Men only. Matched for age. Adjusted for age and smoking. | (Dikshit and Kanhere, 2000)<br>Significant trends for frequency and duration of use |
| Chennai, Tamil Nadu; Bangalore, Karnataka; Trivandrum, Kerala | Oral cavity: ICD 9 codes not specified | Never chewers<br>BQ | 127<br>15 | 232<br>6 | 1.0 (ref.)<br>4.2 (1.5–11.8) | Hospital based, at 3 centers. Matched on center, age and sex. Adjusted for age, center, education, smoking and drinking. | (Balaram *et al.,* 2002)<br>Significant trend for frequency per day |
| Chennai, Tamil Nadu; Trivandrum, Kerala | Oral cavity: ICD 9 codes 140, 141, 143–145 | Non chewers<br>BQ | 122<br>24 | 1471<br>83 | 1.0 (ref)<br>3.4 (2.0–5.7) | Hospital based at 2 centers. Men only. Stratified: only non-smokers and non-drinkers. Adjusted for age, center, education | (Znaor *et al.,* 2003)<br>Significant trends for frequency and duration of use. |
| Trivandrum, Kerala | Oral cavity: ICD 10 codes C001–C009 | Never chewers<br>BQ | 64<br>5 | 561<br>16 | 1.0 (ref.)<br>3.3 (0.9–12.0) | Population based. Matched on age & sex. Adjusted for education, religion, smoking and alcohol drinking (never vs ever). | (Muwonge *et al.,* 2008)<br>Significant trends for frequency and duration of use. |

Contd…

**Table 4.** Areca nut chewing practices ad risk of oral and other head and neck cancers in case control studies in India (Contd…)

| Sex / Location | | Chewing status | Cases | Controls[#] | Odds ratio for oral cancer | Study type. Matching, Adjustments, & Stratifications | Reference |
|---|---|---|---|---|---|---|---|
| **WOMEN** | | | | | | | |
| Bangalore, Karnataka | Oral cavity: ICD sites for lip, tongue (excluding base of the tongue), alveolus, and mouth | Never Chewers<br>BQ | 19<br>9 | 144<br>30 | 1.0 (ref.)<br>2.2 (0.7–6.5) | Hospital based.<br>Matched on age, sex and area of residence. Hospital based.<br>Adjusted for smoking. | (Nandakumar *et al.,* 1990)<br>Significant trends for frequency and duration of chewing in general. |
| Chennai, Bangalore & Trivandrum | Oral cavity: ICD codes not specified | Never Chewers<br>BQ | 29<br>14 | 251<br>5 | 1.0 (ref.)<br>16.4 (4.8–56.5) | Hospital based. Matched on center, age and sex. Adjusted for age, center, education, smoking and drinking. | (Balaram *et al.,* 2002)<br>Significant trends for frequency.<br>Starting chewing at an early age has higher risk only for women. |
| Trivandrum, Kerala | Oral cavity: ICD 10 codes C001–C009 | Never chewers<br>BQ | 16<br>8 | 354<br>28 | 1.0 (ref.)<br>5.4 (2.1–14.1) | Population based. Matched on age & sex. Adjusted for education, religion, smoking and alcohol drinking (never vs. ever) | (Muwonge *et al.,* 2008)<br>Significant trends for frequency and duration of use. |

CI= confidence intervals. BQ=Betel quid without tobacco. Note: Use of lime may be generally inferred even if not mentioned.
BQ= Betel quid; T=tobacco; AN= Areca nut; NS=Not significant; [#]Controls, from voters' list; † Some smokers among chewers.
* $p < 0.001$

without tobacco for men and women combined (Muwonge *et al*., 2008). One study reported an OR of 11.4 for *supari* (areca nut) chewing for men and women combined (Mahapatra *et al.,*2015).

All eight studies had significantly elevated ORs for cancer for chewing of betel quid with tobacco. Trends for frequency were analysed in all but two studies and were significant. Trends for duration analysed in all but three studies and were significant. It is notable that in one study the OR for cancer for past users of any type of betel quid was 11.9 (7.0–20.4), higher than for current users, 4.3 (3.1–6.1) (for men and women combined) (Muwonge *et al.,* 2008), suggesting an accumulation of risk over time before the users quit.

For betel quid with tobacco (BQT), available ORs for men and women combined ranged from 4.8 to 14.6 (Jussawala and Deshpande, 1971; Nandakumar *et al.,* 1990); for men only ORs ranged from from 3.4 to 9.3 (Muwonge *et al.,* 2008; Znaor *et al.,* 2003) and for women only ORs ranged from 30.4 to 45.9 (Nandakumar *et al.* 1990; Balaram *et al.,* 2002), all significant. All of the studies, but one, were matched on age and sex (Table 4). Five studies were adjusted for smoking, and three for alcohol drinking; one study was stratified for smoking and drinking and was of high significance (Znaor *et al.,* 2003).

ORs for areca nut, lime and tobacco use without betel leaf, for men and women combined ranged from a non-significantly elevated 2.4 to a significant 10.2 (Muwonge *et al.,* 2008; Wasnik *et al,* 1998). For women, the only available OR for areca nut, lime and tobacco was 9.1 (Muwonge *et al.,* 2008). An OR for *gutka* for men and women combined was 5.1 and highly significant (Mahapatra *et al.,* 2015).

**Animal Experiments**

Studies in animals carried out to investigate the carcinogenicity of areca nut, its constituents and its products and have helped to validate the results of epidemiologicial studies. Two sets of studies with areca nut (Table 5) and with *pan masala* (Table 6) are reviewed in the following section.

***Areca nut studies***

Three different animal experiments were designed for simultaneous testing of the carcinogenicity of areca nut, in 2–3 months old inbred Swiss mice (n = 65),

**Table 5.** Selected animal experiments on the carcinogenicity of areca nut.

| Animals | Treatment | Route of administration | Frequency | Durations | Cancerous and other changes observed | | Authors, Year, (Country), Notes |
|---|---|---|---|---|---|---|---|
| 25 control<br>Swiss Mice (13M+12F)<br>Aged 2–3 months | Group 1: Pure distilled water | Subcutaneous injections | Once weekly | 10 weeks<br>Then allowed to live their full life span up to 27 months | 0/25; No local tumour | | Ranadive et al., 1976, (India) |
| 40 Experimental<br>Swiss Mice (10 Males & 10 Females)<br>Aged 2–3 months | Aqueous extracts of areca nut:<br><br>Group 1: cold aqueous extract<br>Group 2: hot aqueous extract | Subcutaneous injections | Once weekly | Life span up to 27 months | Fibrosarcomas at the site of injection: (first after 8 months):<br><br>Group 1 (cold) : 10/20<br><br>Group 2 (hot) : 14/20<br>(Equal numbers in both sexes) | | |
| 30 control Golden Syrian hamsters<br>Aged 2–3 months;<br>Sex not recorded | Untreated | Untreated | NA | Killed in two age groups: 6–12 and 13–21 months (e.g. duration from about 3–9 and 10–18 months) | Cheek pouch:<br>0 atypia<br>0 precancers<br>0 cancer | Fore-stomach:<br>0 atypia,<br>1 precancer<br>0 cancer<br>0 glandular stomach ulcerations (GSU) | Ranadive et al., 1979, SEE P 152 IARC (India)<br><br>Results for the two age groups are combined |
| 21 Golden Syrian hamsters;<br>Aged 2–3 months | Aqueous extract of areca nut | Cheek pouches painted inside | Tri-weekly | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch:<br>12 atypia,<br>2 precancers<br>1 cancer | Fore-stomach:<br>5 atypia<br>6 precancers,<br>4 cancers<br>5 GSU | |
| 20 Golden Syrian hamsters;<br>Aged 2–3 months | Polyphenol fraction of areca nut (aqueous) | Cheek pouches painted inside | Tri-weekly | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch:<br>13 atypia<br>6 precancers<br>1 cancer | Fore-stomach:<br>6 atypia<br>2 precancers<br>4 cancers<br>5 GSU | |

Contd…

**Table 5.** Selected animal experiments on the carcinogenicity of areca nut. (Contd…)

| Animals | Treatment | Route of administration | Frequency | Durations | Cancerous and other changes observed | | Authors, Year, (Country), Notes |
|---|---|---|---|---|---|---|---|
| 20 Golden Syrian hamsters; Aged 2–3 months | Whole betel quid aqueous extract | Cheek pouches painted inside | Tri-weekly | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch:<br>11 atypia<br>1 precancers<br>0 cancers | Fore-stomach:<br>2 atypia<br>5 precancers<br>6 cancers<br>4 GSU | Ranadive et al., 1979, SEE P 152 IARC (India)<br><br>Results for the two age groups are combined |
| 13 Golden Syrian hamsters;<br>Aged 2–3 months | Arecanut pieces (to inducet rauma) + aqueous extract of areca nut | Insertion into cheek pouch followed by painting | | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch:<br>8 atypia<br>2 precancer<br>0 cancer | Fore-stomach: 1 atypia<br>2 precancers<br>6 cancers<br>3 GSU | |
| 27 Golden Syrian hamsters;<br>Aged 2–3 months | Market processed suparis (pieces) of two brands (showing combined results) | Insertion into cheek pouch | | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch:<br>5 atypia<br>8 precancers<br>5 cancers | Fore-stomach:<br>5 atypia<br>5 precancers<br>10 cancers<br>4 GSU | |
| 25 Golden Syrian hamsters; Aged 2–3 months | Wax pellet control | Insertion into cheek pouch | Assume every fortnight | Killed in two age groups: 6–12 and 13–21 months | No cancerous changes/lesions | | |
| 18 Golden Syrian hamsters; Aged 2–3 months | Wax Pellets containing Betel quid | Insertion into cheek pouch | Every fortnight | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch:<br>3 atypia<br>1 precancers<br>4 cancers | Fore-stomach: 3 atypia<br>1 precancers<br>8 cancers<br>4 GSU | |

Contd…

**Table 5.** Selected animal experiments on the carcinogenicity of areca nut. (Contd…)

| Animals | Treatment | Route of administration | Frequency | Durations | Cancerous and other changes observed | | Authors, Year, (Country), Notes |
|---|---|---|---|---|---|---|---|
| 9 Golden Syrian hamsters; Aged 2–3 months | Gelatine capsule control | Insertion into cheek pouch | Assume every fortnight | Killed in two age groups: 6–12 and 13–21 months | No cancerous changes/lesions | | Ranadive et al., 1979, SEE P 152 IARC (India) |
| 19 Golden Syrian hamsters; Aged 2–3 months | Gelatine capsules containing Areca Nut powder | Insertion into cheek pouch | Every fortnight | Killed in two age groups: 6–12 and 13–21 months | Cheek pouch: 5 atypia, 7 precancer, 4 cancers | Fore-stomach: 4 atypia 1 precancers 6 cancers 1 GSU | Results for the two age groups are combined |
| 15 Golden Syrian hamsters; Aged 2–3 months | DMBA wax pellet Standard carcinogen control | Cheek pouch | Assume every fortnight | Killed after 6–12 months | Cheek pouch: 0 atypia, 3 precancer, 12 cancer | Fore-stomach: 4 atypia 1 precancers 6 cancers 2 GSU | |
| 20 control albino BALB-C mice | Normal saline solution | Buccal mucosa via pipette | Twice daily; 6 days a week | Group 1: 300 days Group 2: 350 days Group 3: 450 days Group 4: 600 days | None (normal status) Mean body weight at 600 days: 49.3 g ± 4.7 g | | Perera et al., 2007, (Sri Lanka) |
| 20 albino BALB-C mice in 4 subgroups of 5 mice each; Aged 12 weeks | Aqueous extracts of areca nut | Buccal mucosa via pipette | Twice daily; 6 days a week | Group 1: 300 days Group 2: 350 days Group 3: 450 days Group 4: 600 days | Cellularity, inflammation and muscle atrophy increased from normal to mild by 300 days and remained so up to 600 days. Compared with controls at 600 days the difference was significant (Wilcoxon statistic 15; P = 0.03) Mean body weight at 600 days: 44.5 g. ± 2.8 g | | The lower average body weight of the exposed mice compared to controls was noted. |

DMBA = 7, 12 - dimethylebenz (a) anthracene.

**Table 6:** Selected animal experiments on the carcinogenicity of *pan masala*, a processed from of areca nut.

| Animals | Treatment | Treatment Location | Frequency | Durations | Changes observed | Authors, Year, (Country), Notes |
|---|---|---|---|---|---|---|
| 14 Albino Wistar rats (Controls) | No treatment | --- | --- | 8 months | No significant changes in biopsies of buccal mucosa taken at beginning and end of 8 month study period | Khrime et al., 1991 (India) |
| 21 Albino Wistar rats | Paste made of *pan masala* | Oral cavity | Alternate days | 6 months | Dysplasia in 65% of animals; and thickened & condensed submucosal collagen seen in 88% biopsies | |
| 20 Swiss mice (10 Males; 10 Females) of S/RVCri strain, Aged 6–7 weeks (Controls) | Normal diet | Oral consumption | Daily | For intermediate period: killed at 6, 12 and 18 months | No neoplastic lesions | Bhisey et al., 1999 (India) |
| 40 Swiss mice (30 M; 30 F) of S/RVCri strain, Aged 6–7 weeks | 2 dose groups: Powdered *pan masala* mixed in feed at concentrations of 2.5% or 5% | Oral Consumption (10/ gender/ dose group) | Daily | For intermediate period: killed at 6, 12 and 18 months | 2.5% *pan masala* group:<br>• No tumors.<br>• Forestomach hyperplasia in 10 out of 60 mice<br>5.0% *pan masala* group:<br>• Adenocarcinoma of the lung in 1 male and 1 female.<br>• Forestomach hyperplasia in 5 out of 60 mice | |
| 108 Swiss mice (54 Males, 54 Females) of S/RVCri strain, Aged 6–7 weeks (Controls) | Normal diet | Oral Consumption (54/gender/ dose group) | Daily | For lifetime: killed when moribund or at 24 months | No neoplastic lesions | |
| 216 Swiss mice (108 M; 108 F) of the S/RVCri strain, Aged 6–7 weeks | 2 does groups: *Pan masala* finely powdered, mixed with feed at concentrations of 2.5% & 5% and pelleted. (54 mice of each sex allocated to each dose = 108) | Oral Consumption (54/gender/ dose group) | Daily | For lifetime: killed when moribund or at 24 months | 2.5% *pan masala* group:<br>• 5 malignant lesions<br>5.0% *pan masala* group:<br>• 7 malignant lesions<br>Overall 15 benign lesions and 12 malignant lesions Decrease in survival as shown by log rank test (p = 0.02). Lung adenocarcinoma, showed a 2-fold increase in the higher dose group compared to lower dose group. | |

NA = Not applicable; DMSO= Dimethyl Sulphoxide; DMBA = 7-1 2-Dimethyl-benz(a)anthracene

C17 mice (n = 78), and golden hamsters (n = 45) (Table 5) (Ranadive *et al.,* 1976). Hot and cold aqueous solutions of areca nut were injected subcutaneously in Swiss mice, once a week. Control groups of animals were treated with distilled water. In C17 mice and golden hamsters, dimethyl sulfoxide (DMSO) solutions were used with the aim of enhancing the dermal absorption of the areca nut components from the extract. DMSO areca nut solutions were applied on the skin of the backs of the C17 mice thrice weekly between the shoulder blades. Control groups of animals were treated with 100% DMSO. The hamsters received DMSO areca nut solutions, painted inside the cheek pouch three times a week.

By the end of the lifespan of the Swiss mice (≤ 27 months), ten of 20 mice subcutaneously injected with cold water areca nut extract developed transplantable fibrosarcomas (50%) at the site of injection and 14 of 20 injected with hot water areca nut extract developed fibrosarcomas (Table 5). Tumours were not observed in the internal organs of control and experimental Swiss mice. Skin applications of DMSO areca nut extracts in C17 mice up to 27 months resulted in some mild to moderate hyperplasia but no skin lesions. Cheek pouches of golden hamsters painted with DMSO extract of areca nut showed some early malignant changes (atypia) up to 24 months. The authors concluded that areca nut demonstrated a carcinogenic principle using aqueous extracts (Ranadive *et al.,* 1976).

With the insights gained, a subsequent set of experiments was conducted in Golden Syrian hamsters. Hamster cheek pouches were painted with aqueous areca nut or betel quid extracts or distilled water for controls. Besides, either wax pellets or gelatin capsules containing betel quid or areca nut powder, or pieces of commercially processed supari were inserted into the cheek pouches and compared to controls with distilled water filled wax pellets or empty gelatine capsules on a triweekly basis. In contrast to the control groups, all treated groups developed numerous malignant changes and cancers (Table 5), a majority occurring in the forestomach (Ranadive *et al.,* 1979).

In a recent study in Sri Lanka, 20 BALB-C mice treated with aqueous extract of fresh areca nut for a maximum of 600 days, developed OSF-like condition in the buccal mucosa with 20 mice treated with normal saline solution as control

groups (Table 5). The changes observed in oral tissues of the mice included proliferation of fibroblasts (increased cellularity), abundance of collagen fibres, increased thickness of the *lamina propria*, infiltration of inflammatory cells (mainly lymphocytes and plasma cells) in the connective tissue, and atrophic epithelium and muscle atrophy in the submucosal layer. These changes closely resembled the human oral mucosa affected with OSF (Perera *et al.,* 2007).

***Pan masala***

The histopathological changes due to *pan masala* were depicted in a study on painting a paste of a well-known brand of *pan masala* in the oral cavity of 21 albino rats on alternate days for six months. Mild to moderate loss of nuclear polarity and increased keratosis and parakeratosis, inflammatory cell infiltration and vascularity were observed (Table 6). Nearly eight out of nine biopsies showed thickened and condensed sub-mucosal collagen. Thus, histopathological changes observed were similar to OSF in humans (Khrime *et al.,* 1991). Further, carcinogenicity of *pan masala* was studied in six groups each of 54 Swiss mice (three groups of males and three groups of females, 6–7 weeks of age). The mice were fed diet containing either dry finely powdered *pan masala* (2.5% or 5%), or normal diet (control group) either for life or an intermediate period. The animals were sacrificed when moribund or after 24 months, whichever was earlier. In the intermediate period group, no tumours were seen in the group fed with 2.5% *pan masala*, but two mice in the 5% *pan masala* group developed adenocarcinoma of the lung. In the lifetime group, a total of 15 benign and 12 malignant tumours were observed in the treated mice, while no tumours were found in controls. Most of the malignant tumours occurred in the liver (n = 13), lung (n = 8) and stomach (n = 3). The most common lung neoplasm was lung adenocarcinoma. The mice fed *pan masala* also lost weight after six months and lived a significantly shorter life span compared to the control mice. Thus, the authors have demonstrated evidence of the carcinogencity of *pan masala* in different mouse tissues, indicating that *pan masala* should be considered a potential human carcinogen (Bhisey *et al.,* 1999).

**Mechanistic Evidence of Carcinogenicity**

The causal biochemical and molecular mechanisms of oral submucous fibrosis and oral cancer in areca nut chewers are broadly summarized here. During chewing, certain areca nut components, including the alkaloids (mainly arecoline and arecaidine) and polyphenols (tannins, flavonols and catechins) are absorbed through the oral mucosa into the tissues and blood stream (IARC, 2004). These components promote simultaneous abnormal changes in the two main layers of the oral mucosa. A role of in areca nut metabolites in stimulating collagen synthesis in oral mucosa was suggested by tissue culture studies on human fibroblasts from the oral mucosa (Canniff and Harvey, 1981; Harvey *et al.,* 1986; Murti *et al.,* 1995). In the presence of slaked lime (aqueous calcium hydroxide), arecoline, the principal alkaloid, is hydrolysed into arecaidine resulting in irritation and induction of inflammatory mediators (Feller *et al.,* 2013), followed by inflammation. This inflammation stimulates fibroblast proliferation in the *lamina propria*, the connective tissue layer of the mucosa. The stimulated fibroblasts then synthesize excess collagen fibres, resulting in dense fibrosis, leading to stiffening of the mucosa and eventually to palpable fibrous bands. The increasing atrophy of the overlying epithelium, leads to a burning sensation, impaired vasculature and ulcerations (Angadi and Rao, 2011; Khan *et al.,* 2012). Leukoplakia caused by areca nut may cause atrophy (Borle, 2014). Impaired vasculature is initially responsible for the whitish appearance or blanching of the mucosa due to reduced blood supply, occurring from an early stage of the disease prior to fibrous bands appearance (Ekanayaka and Tilakaratne, 2013). The polyphenols and arecoline react in the presence of slaked lime, forming reactive oxygen species, such as the hydroxyl radical (Nair *et al.,* 1995), resulting in inhibition of collagenase enzymes and phagocytosis, preventing collagen degradation and increasing fibrosis. The high copper content of areca nut participates in promoting fibrogenesis (Angadi and Rao, 2011; Khan *et al.,* 2012).

Genetic damage is observed in the oral mucosa of areca nut chewers. Areca nut-specific nitrosamines, or their precursors, and reactive oxygen species generated in the saliva during betel quid chewing are implicated in causing various forms of

genetic damage in the keratinocytes of the basal layer. The copper content promotes formation of cross linkages between the fibrous bands (Angadi and Rao, 2011; Khan *et al*., 2012). Betel leaf contains substances, including beta carotene that functions as scavenger of reactive oxygen species and help prevents DNA breakage, thus lowering the risk of cancer among *pan* chewers, compared to those who chew areca nut or its products without betel leaf (Jeng *et al.,* 2002). Genetic damage is indicated by micronucleated cells in the exfoliated oral epithelial cells of chewers of areca nut products and OSF patients (Desai *et al.,* 1996). Micronucleated cells in chewers are in excess ($p < 0.0001$) of those in non-chewers (Joshi *et al.,* 2011). Further genetic alterations in the keratinocytes followed by increased proliferation may lead to malignant phenotypes. A higher percentage of cells with karyolysis (dissolution of chromatin or nuclear contents) has demonstrated in OSF ($p < 0.05$) compared to non-chewers (Joshi *et al.,* 2011). Interactions between the fibroblasts and the keratinocytes appear to promote malignant transformation in OSF (Ekanayaka *et al.,* 2013).

Nitrosation of the areca nut alkaloids occurs in saliva in the presence of bacterial enzymes, particularly in individuals with poor oral hygiene. The resulting areca nut specific nitrosamines are mutagenic and form DNA adducts in experimental systems, indicating cancer risk (IARC, 2004). Aflatoxins, in areca nut due to fungus infection, form DNA adducts (IARC, 2004). The various genetic lesions (adducts, breaks, etc.) that form with the use of areca nut may progress to cancer over longer time periods (Shah *et al.,* 2012).

## DISCUSSION

There is convincing evidence that betel quid or areca nut chewing without tobacco is a cause of oral cancer. A meta-analysis of case-control studies over the last 50 years, on oral/oropharyngeal cancers concluded that overall estimate of relative risk (RR) for use of betel quid without tobacco in the Indian subcontinent was 2.6 (95%CI: 2.0–3.3) (Guha *et al.,* 2014). The frequency of use per day was a more important factor than duration of the habit was unequivocally shown in OSF. The chewing of betel quid containing tobacco confers a greater risk than chewing betel quid without tobacco, besides the added carcinogenicity of tobacco.

Comparing ORs for use of different products and duration of use showed significant differences in risk for OSF. The betel quid chewers were diagnosed after 6–10 years of chewing, whereas *pan masala* and or *gutka* chewers presented with OSF after 2–3 years of use. Thus, chewing of *pan masala* and/or *gutka* causes progression to OSF faster than betel quid. The possible reasons considered were absence of betel leaf and higher consumption by weight of areca nut (Babu *et al.,* 1996).

In the Mumbai Cohort Study the RRs for mortality due to oral and pharyngeal cancers for areca nut or betel quid chewing without or with tobacco did not show significantly elevated RRs, although an RR was significant for other forms of smokeless tobacco use (Gupta *et al.,* 2005). The analysis of incident cancers in the Mumbai Cohort Study (Pednekar *et al.,* 2011), RRs reported for all cancers combined were elevated but not significantly for use of betel quid or areca nut; while RR for cancer of the oral cavity and pharynx for all smokeless tobacco use combined was significant (RR 1.48, 95% CI: 1.03–2.13). These results may in part be due to the rare use of areca nut and tobacco without betel leaf, protective effect of betel leaf among betel quid users, and number of person years in the cohort yielding a small number of cancer cases during the study period. In contrast, in case-control studies, cancer patients come to specialised treatment centres from very wide geographical areas, home to very large populations.

Risk estimates for precancerous lesions and cancer among the exposed are significantly elevated in case control studies, showing strength of association and a temporal relationship. Most case control studies on OSF or cancer show a dose response relationship with higher frequency per day and greater duration of use. The observed changes in exposed animals and humans fit broadly within known pathways for carcinogenesis, including chronic inflammation and genetic damage, showing plausibility and coherence of findings. Changes in the cheek mucosa occur where the quid is kept by areca nut chewers, implying a direct association. OSF shows specificity to areca nut use, almost always preceding mouth cancer in areca nut users. Alternate explanations, such as the consumption of chillies alcohol or tobacco are not causally related to OSF or cancer. Data showing a positive correlation in OSF and current

users of only tobacco are not verified and past use of areca nut not known. Thus the evidence described in this review is abundantly clear and unequivocally fits the Bradford Hill criteria for causality (Hill, 1965). Policy decisions by the Indian government to control the use of areca nut for the benefit of public health are the need of the hour.

The increasing prevalence of use of areca nut products containing tobacco such as *gutka, mawa,* and *pan masala* coincides with rise in OSF and oral cancer primarily at the site of placement in the buccal mucosa. Hospitals in India have noticed increase in admissions for OSF and oral cancer from patients using areca nut products. Thus, convincing evidence on the carcinogenicity of areca nut and tobacco, common use and consequent hazards are obvious in the Indian context. Besides, it is alarming that areca nut products are increasingly exported (40 countries and more), with official quantities of export tripling since 1991 (Kammardi *et al.,* 2012). While tobacco has been widely recognised as a carcinogen, carcinogenicity of areca nut has not been widely communicated or acknowledged. It is mandatory to dispel ignorance of the hazards of areca nut and recognize the importance of increasing awareness of the carcinogenic potential of areca nut.

## CONCLUSIONS

In view of the elevated risk of cancer posed by use of areca nut and the rising incidence of OSF and oral cancer in India, control of areca nut and its products, through banning, is justified in order to contain the adverse health effects on the population and improve public health in the affected individuals. In addition, appropriate communications programmes on the harmfulness of areca nut are strongly recommended.

## CONFLICT OF INTEREST

The authors acknowledge no conflict of interest.

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Review

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# Identification of Therapeutic Targets for Cancer: Proteomic Technologies and Strategies are the Key to Success

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With the emergence of the field of 'omics' a new era of systematic global profiling of cellular molecules has been initiated in biology. Different 'omics' approaches have been extensively used to identify biomarkers for better diagnosis and prognosis, therapeutic strategies and monitoring response to therapy in diverse types of cancers. Proteomics is the approach of choice for identification of therapeutic targets. This is because therapeutic modulation of expression, post-translational modification and activity of a protein can directly rectify the derangement in the disease-causing cellular pathway. The current review scans literature on tumor proteomics to understand the influence of developments in proteomics technology and study approaches on identification of targets for therapy. Diversity of tumor types, molecular heterogeneity in pathologically indistinguishable tumors provides ample challenge to assess the strength of proteomics in identification of drug targets. The review highlights comparative proteomic profiling by gel-based or gel free approach, in tumor and normal tissues or chemo-resistant/sensitive tumor tissues have identified differentiator proteins, with potential as targetsas therapeutic targets. Further, along with evolution in proteomic technologies for identification and quantification of proteins, various tools for functional analysis of proteins have contributed to strategies for target identification. It also suggests that future advances in quantitative, functional and structural proteomics isare necessary to widen the search for therapeutic targets.

## INTRODUCTION

The pace of development of technology is a rate determining factor in the rapid progress of basic and applied sciences. This is exemplified in the healthcare sector by the translational advances of the technological inventions in the healthcare sectorwhich include computerized tomography (CT) and magnetic resonance imaging (MRI) for diagnosis; laparoscopes and cardiopulmonary bypass pumps for surgery; and systems for therapies such as radiotherapy and dialysis. Apart from these technologies which are directly used in clinics,

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advances in optical and analytical instruments have revolutionized biomedical research contributing to the progress in understanding pathobiology of diseases and their management. The current review focuses on proteomic technology-driven advances in the identification of therapeutic targets in various cancers, as a milestone in development of targeted therapeutics.

**Serendipitous discoveries of drugs and drug targets**

Identification of novel therapeutic targets remains an area of interest to clinicians and biotechnology and pharmaceutical industries. Several drugs have been discovered earlier by extraction of the active principle from natural sources such as plants traditionally used to treat a disease (Cragg, 2013). A few drugs were discovered by serendipity, a classic example being penicillin from a contaminating fungus (Bennet, 2001) and a cancer chemotherapeutic vincristine by its undesired myelosuppressive effect when used to treat diabetes (Johnson, 1963). With improved understanding of molecular machinery of the cell and aberrations associated with disease conditions, efforts were made to design drugs to target the disease-causing molecule. This approach was favoured for diseases with an established causal association with a molecular alteration. For example, in chronic myeloid leukemia (CML), inhibition of the transforming tyrosine kinase has been an excellent targeted therapy in CML patients (Freireich, 2014). However, for a long time, serendipity remained the basis for discoveries of drugs as well as for the identification of targets as in CML. Such discoveries require fortuitous co-occurrence of the phenomenon which led to the discovery and an alert analyst. A structured approach is necessary for identification of therapeutic targets and discovery of new drugs.

**'Omics' for systematic identification of molecular alterations in tumors**

With emergence of 'Omics', biology began a new era of planned and systematic search for global molecular alterations in diseases. The strength of this approach is evident from its potential to unravel key molecular events from the numerous molecular alterations seen in cancer (Bertrand, 2015; Castro-Vega, 2015). Multifactorial and multigenic origin of cancer is reflected in the diverse molecular alterations in pathologically indistinguishable tumors, which progress and respond differentially to drugs and therefore require an individualized therapeutic approach. Interrogation of differences in genome, transciptome, proteome, metabolome, lipidome have led to the identification of several cancer biomarkers useful in diagnosis (Du, 2014; Liu, 2015), predict disease prognosis (Minca, 2014; Shipitsin, 2014), assist in choice of therapy (Fėnichel, 2014; Sjøholt,

2006) and monitor response to therapy (Rebecca, 2014).

**Proteomics: The 'Omics' of choice for identification of drug targets**

Proteins are functional molecules in a cell and alterations in their function can affect cell phenotype. Proteomics, a global study of proteins, is therefore an approach of choice to identify drug targets. The study of a cell proteome is challenging due to the complexity of protein structure; effect of post-translational processing i.e., cleavage or modification of function, sub-cellular localization and changes in interacting molecules including substrates (Parker, 2014); dynamic range of proteins expressed in a cell (Corthals, 2000) and the temporal variations in the proteins.all the above variables. In a disease state, alterations in any of these parameters may occur in one to several proteins. Challenges posed by these compounding factors to proteomic profiling have been addressed at every step of development of proteomic technology and supported by advances in the fields of genomics (Wang, 2014), bioinformatics (Boguski, 2003) and computational biology (Dowsey, 2003).

**Technological advances meet the challenges of profiling complex proteomes**

***Protein identification by mass spectrometry***

Emergence of the field of proteomics began with the changes improvisations in mass spectrometry (MS) instrumentation and techniques in late 1990s. Proteins and other biomolecules fragmented by the previously used ionization methods, were preserved intact by the softer ionizations – electrospray ionization (ESI) (Karas, 1988) and matrix assisted laser desorption ionization (MALDI) (Fenn, 1989) promoting MS as a tool for biologists. The ionizers were coupled to an analyzer, such as a quadrupole, time of flight, ion trap, etc. which separated the ionized molecules on the basis of their mass/charge (m/z) ratio. MS-based identification of proteins was achieved by comparing the masses of peptides generated by cleavage of a protein using specific protease and those generated 'in silico' by digestion of sequences available in public databases with the same protease, an application called peptide mass fingerprinting (PMF). In tandem mass spectrometry platforms (MS-MS) with more than one analyzer, peptides detected in the first analyzer are put through controlled fragmentation by collision induced dissociation (CID). The accurate masses of the peptide fragments obtained from analysis in the second analyzer, when processed by appropriate software designedgenerated the sequence of the peptide. The sequence is used for protein identification. Thus mass spectrometry circumvented the need for a probe to detect the protein of interest from a complex mixture of proteins (Abersold, 2003). Further, deeper interrogation of the proteome, essential for biomarker

discovery, was made possible by the features in mass spectrometer which allowed data-independent fragment analysis (Sajic, 2014). Further advancement in the fragmentation mechanisms, introduction of electron transfer dissociation (ETD), improved detectability of labile post-translational modifications in proteins (Kim, 2012). Advances in mass spectrometry therefore improved the detection, identification and knowledge of the post-translational modifications in proteins.

***Reduction in protein complexity before mass spectrometry***

The strength of mass spectrometry for protein identification is compromised in biological samples due to the complexity of proteome and dynamic range of protein expression. High or medium abundant species in a sample may interfere with the detection of low abundant species, called "suppression effect". Therefore to enable identification of less abundant proteins and to enrich less concentrated species, clinical proteomics studies require fractionation of proteins from complex mixtures, prior to proceeding for identification by mass spectrometry analysis. The strategy which can optimally reduce the complexity would differ in each sample (Camerini, 2015).

Among gel based separation methods, two dimensional gel electrophoresis (2DGE) has been the method of choice. Separated proteins are subject to in-gel digestion with specific proteases, peptides are extracted from the gel and subjected to MS for protein identification. The technique of 2DGE has metamorphosed due to availability of immobilized pH gradients (IPG), multi-gel electrophoresis apparatus improving the reproducibility of profiles, and staining protocols using fluorescent dyes with improved sensitivity and linearity over a wider dynamic concentration range. IPG strips are available in micro pH ranges for wider resolution, and thereby improving detection of proteins (Gorg, 2000). Apart from 2DGE, capillary electrophoresis and agarose gel isoelectric focusing are used to separate proteins from biological samples (Manabe, 2000).

Liquid chromatography (LC) is a versatile method for protein separation as different column chemistries allow separation of proteins based on distinct characteristics (Di Palma, 2012). Since ESI can ionize samples introduced in liquid phase, LC-MS platforms were designed, wherein sample separated on LC can be introduced directly into the ionizer of MS. However, as biological samples are obtained in limiting amounts, efforts have been made to increase the sensitivity of detection by tapping measures beyond the improvisations in the hardware of MS. In LC-MS systems, reducing the flow rate contributes to higher overall sensitivities due to the higher efficiencies in ESI and reduced ion suppression effects (Köcher, 2014). Nano LC-MS-MS is the

configuration of choice for biological samples analysis.

***Separation and identification tools for differential quantification***

Distinction between expression of proteins in disease and normal state requires quantitative evaluation of the expression. Several tools are available to achieve the same. Quantitative proteomics can be categorized into absolute and relative types. Absolute quantitation determines changes in protein expression in terms of an exact amount or concentration of each the protein present; whereas relative quantitation determines the up- or down-regulation of a protein relative to the control sample, generally used in clinical proteomics. In MS based quantitation, the relative concentration can be obtained by: Label free methods are based on less rigorous mass spectrometry, with more reliance on bioinformatics and separation techniques. Chemical labeling is applicable to a wider range of biological samples, and methods such as isotope coded affinity tags (iCAT) and isobaric tags for relative and absolute quantitation (iTRAQ), are favoured in quantitative proteomics. The control and test samples are labeled with separate tags, and intensity of the same ion with distinct tags indicates the relative quantitation (Elliott, 2009). Protein quantitation can be done by two dimensional difference gel electrophoresis (2D-DIGE) wherein proteins from different samples are labeled using dyes that provide different fluorescence wavelengths for detection. The labeled samples to be quantitated are mixed in equal proportion and separated on the same gel. The gels are scanned and the relative fluorescence of distinct dyes is recorded for quantitation (Timms, 2008). The quantitative differences obtained from the above mentioned studies are the first scan of the differentiators, which are the storehouse of potential biomarkers and therapeutic targets. .

***Selection of appropriate proteomic technology***

Advocates of gel free and gel-based proteomics favour the method of choice due to certain advantages. Shot-gun proteomics carried out using LC-MS platforms are less laborious, more reproducible and capable of generating a larger profile (Wilkins, 2009). On the other hand, gel-based proteomics (Rogowska-Wrzesinska, 2013) aids identification and sequencing of proteins from organisms with minimal genomic information, efficiently identifies protein isoforms and proteins modified by glycosylation, proteolytic cleavage, etc.

**Selection of comparison groups for identification of therapeutic targets**

Identification of differentiators has been carried out with different aims and therefore differentiators are derived from diverse comparison groups. However, they inadvertently point at the key

molecules as potential therapeutic targets.

***Comparison of tumor and normal in retrospective or prospective studies***

For understanding cancer biology, the profile of differentiators are generated to reveal molecular mechanisms responsible for disease progression. Potential markers for progression of pulmonary squamous cell carcinoma were identified by examining samples of lung SCC and adjacent normal tissues using 2D-DIGE (Lihong, 2014). Markers of progression of oral squamous cell carcinoma from premaliganant lesion to carcinoma have been similarly demonstrated (Wang, 2009). Using gel free approach, insight into the underlying mechanisms of formation of polyploidy giant cancer cells (PGCC) and the relationship between PGCCs and cancer stem cells in patients with ovarian cancers has been established (Zhang, 2013).

Differential molecular profile is often generated for better stratification of tumors in order to improve diagnosis and management of the disease. Diagnostic markers have been identified by 2D-DIGE for cervical cancer (Canales, 2014; Guo, 2014). Similarly, using gel-based approach it has been shown that high expression level of Galectin-1 may correlate with development of nasopharyngeal carcinoma (NPC), and Galectin-1 as a potential diagnostic marker or therapeutic target for NPC (Tang, 2010). Further, a significant proportion of primary breast cancers are negative for estrogen receptors (ER), progesterone receptor (PgR), and Her2, comprising the triple negative breast cancer (TNBC) group. Women with TNBC have poor prognosis because of the aggressive nature of the tumors and current lack of suitable targeted therapies. The increased expression of Mage-A4 in the tumors enabled the detection of the protein in the tumor interstitial fluids and in sera. Immunotherapeutics approaches specifically targeted Mage-A4 protein, or Mage-A protein family members represents novel management options for TNBC (Cabezon, 2013).

An alternative aim for profiling of tumors is to predict prognosis. Using gel based proteomics approach, a subgroup of breast tumors with overexpressed C7 or f24 showed poor clinical outcome (Gromov, 2010). Similarly, LC-MS approach identified RBBP6 as prognostic marker for gastric cancer stem cell (Morisaki, 2014) and WD repeat containing protein 1 was identified as a diagnostic marker in the interstitial fluid from ovarian cancer (Haslene-Hox, 2013).

***Comparison groups with focus on therapeutics***

Several differentiators identified earlier may be useful potential therapeutic targets. Additionally, proteomic analyses aimed at understanding mechanism of drug resistance or drug action have greater probability to identify as therapeutic

targets.

Analysis of cell lines or samples from patients, untreated or treated with a drug, provides insight into the molecular mechanism of action of drugs/ chemo-preventive agents. Further identification of the drug modulated pathway may indicate therapeutic targets for further exploration. Curcumin, a natural anticancer agent, inhibits cell growth in a number of tumor cell lines and animal models. Molecular mechanism of curcumin induced apoptosis in different gastric cancer cell lines was studied by 2DGE (Cai, 2013). Similarly, using gel free approach, *Bifidobacterium* infantis thymidine kinase/ nucleoside analogue ganciclovir (BI-TK/ GCV) exhibited sustainable anti-tumor growth activity and induced apoptosis in bladder cancer, via peroxiredoxin I and NF kB pathway (Jiang, 2014).

Comparison of chemo-sensitive and -resistant tumors/cell lines provides information of the molecular basis of resistance and hence molecules to be considered as alternate therapeutic targets. In mantle cell lymphoma, a rare aggressive type of B cell non-Hodgkin's lymphoma, wherein response to chemotherapy tends to be short and patients relapse, the tumor necrosis factor related apoptosis inducing ligand (TRAIL) is a novel molecule with antitumor effects. In TRAIL resistant cases, 2DGE analysis demonstrated downregulation of the key enzymes of purine metabolism with profound effects on nucleotide homeostasis and can render cells vulnerable to further disruption of purine nucleotide metabolism. Thus proteins in this e pathway identifiedare potential therapeutic targets for selective elimination of resistant cells (Pospisilova, 2013). Chemo-resistance hinders effective treatment in several human cancers. HSP27 is as an alternate target for anticancer drug developmentin gemcitabine therapy resistant pancreatic cancer (Liu, 2012). Histone deacetylase inhibitors (HDACi) demonstrates anticancer activities and used in combination therapy. In lymphoid cell lines, 2DGE analysis has identified HSPA1A as an overexpression with resistance to valproic acid HDAC inhibitor. *In vitro* experiments demonstrate that treatment with KNK-437, an inhibitor of HSPA1A, enhanced cytotoxic effects of valproic acid, thereby identifying HSPA1A as a possible therapeutic target, in combination with HDACi, for lymphoid neoplasms (Fuji, 2012).

**Authentication of the potential of identified therapeutic target**

Differentiators have been identified for several cancers, however, differentiators as a therapeutic targets needs further investigations. Bioinformatic tools for pathway identification are extensively used to find a functional link between the differentiators. A molecule in a pathway associated with hallmarks of cancer

(Hanahan, 2011), qualify as potential therapeutic targets. However, the potential needs to be authenticated experimentally. In several studies, over expression or activation as well as down-regulation or inhibition of the identified potential drug target is used to demonstrate the effect on tumor promotion or progression.

Tamoxifen (Tam) is a widely used selective estrogen receptor modulator (SERM) for treatment of hormone-responsive breast cancer and acts via inhibition of E6AP expression identified as a differentiator by 2DGE. Authentication of E6AP as a therapeutic target was achieved by demonstration of Tam- and siE6AP-mediated inhibition of E6AP with subsequent enhanced G0-G1 growth arrest and apoptosis (Lochab, 2012). Small interfering RNA (siRNA)-mediated knockdown confirmed a functional role for MDA-9 and GRP78 in promoting cell invasion in A375 cells (Guan, 2014). Similarly in liposarcoma, an aggressive cancer with poor outcome, gankyrin oncoprotein showed a significantly high expression. Inhibition of gankyrin led to reduction of *in vitro* cell proliferation, colony-formation and migration, besides *in vivo* dedifferentiated liposarcoma tumorigenesis (Hwang, 2014). KvLQT1 channel blockade was showed efficient reduction of A549 and H460 cell proliferation and migration. Moreover, KvLQT1 overexpression in AD samples suggested it to be a potential therapeutic target in lung cancer (Girault, 2014). In an *ex vivo* model, siRNA mediated inhibition of HSP70, showed dose-dependent inhibition of cell growth and burst formation unit erythroid (BFU-E), increased apoptosis in the erythroid lineage and decreased pJAK2 signaling. Thus HSP70 as a potential therapeutic target in myeloproliferative neoplasms especially polycythemia vera was confirmed (Gallardo, 2013). Similarly inhibition of Apg-2 showed decreased cell proliferation and induced apoptosis in BCR/ABL positive cells, indicating an additional therapeutic target for chronic myeloid leukemia (Li, 2013). Whereas, in chronic lymphocytic leukemia (CLL) a clonal malignancy with immense clinical heterogeneity with variable prognosis, hyper reactivity of the B cell receptor (BCR) to unknown antigen ligation plays a pivotal role in CLL survival. Proteomic analysis revealed that kininogen, a critical protein of kinin-kallikrein system, was upregulated upon BCR stimulation and mayprovide a therapeutic target in CLL (Kashuba, 2013). Further, in MLL-rearranged leukemia, TET1 was identified as a potential therapeutic target (Huang, 2013). Similarly in endometrial cancer, overexpression of bone marrow stromal antigen 2 (BST2) was detected in LC-MS analysis and confirmed by immuno-histochemistry using clinical samples. In an *in vivo* xenograft model, BST2 antibody treatment inhibited tumor growth of BST2-positive endometrial cancer cells in an NK cell-dependent manner,

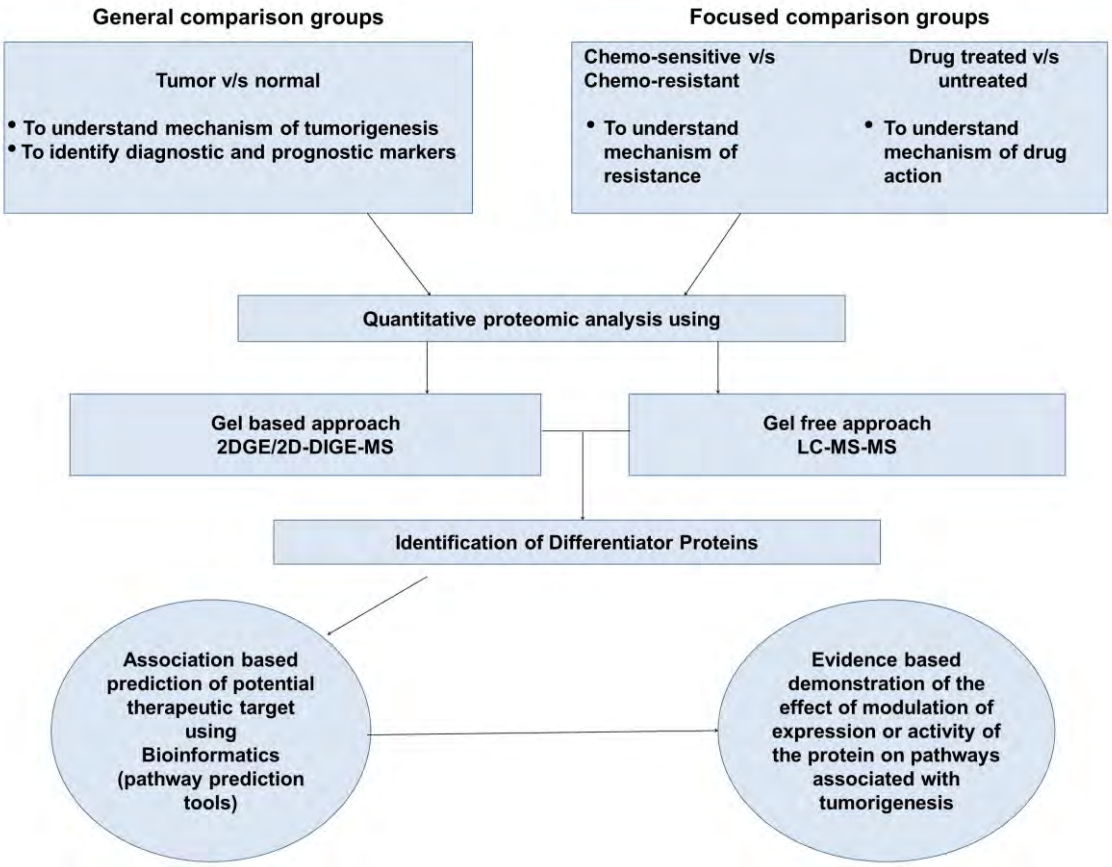


**Figure 1:** Various strategies and technologies for proteomic identification of therapeutic targets for cancer. (2D-DIGE: two dimensional difference gel electrophoresis; MS- mass spectrometry).

advocating candidacy of BST2 as a therapeutic target (Yokoyama, 2013). These studies provide an initial authentication of the potential of identified therapeutic targets.

To summarize, the review highlights usefulness of proteomic technology in identification of therapeutic targets as outlined in figure 1. The review reveals that differentiators, identified by both gel-based and gel free approaches qualify therapeutic targets. It appears that comparative proteomic analysis of chemosensitive and chemoresistant cells as well as that of drug treated and untreated cells, are useful in identification of therapeutic targets. The search strategy for therapeutic targets has evolved from association based approaches wherein a differentiator protein with known role in key functional pathways qualified as a potential target. Evidence based selection of therapeutic targets necessitated experimental demonstration of the ability of the differentiator to affect the hallmarks of cancer (Guo, 2013). Thus, we conclude that advances in proteomic technology and refinements in experimental strategies have contributed to identification of therapeutic targets in tumors and in turn to the field of targeted therapeutics.

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Review

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# Genetic Markers and Evolution of Targeted Therapy in Cancer

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The advances in biotechnology including high throughput platforms, and bioinformatics has resulted in detailing molecular pathology of various cancers, identifying targets such as fusion genes, chimeric RNA, fusion proteins, amplified gene, genes with point mutation, overexpression or down regulation of RNA, microRNA (miRNA) and aberrant DNA methylation. The genetic markers provide diagnostic, prognostic and therapeutic markers, and may also provide predictive markers. Several targeted molecules have been identified as cell surface antigens and tyrosine kinases e. g. FLT3, NPM1, CEBPA and PRAM1 in acute myeloid leukemia (AML); BCR-ABL1 in chronic myeloid leukemia; JAK2 in chronic myeloproliferative disorders; ALK, EGFR, K-RAS and BRAF in lung cancer; BRAF, KIT in melanoma; HER2 in breast cancer. The driver molecules and their mechanism of actions revealed various oncogenic pathways in the development of effective inhibitor molecules/proteins as targeted therapy, and novel mutations in the genes associated with the inhibitor protein. Targeted cancer therapy aimed to antagonize the deregulated molecule/s, commonly comprises therapeutic monoclonal antibodies and small molecule inhibitors. *In vitro* studies and clinical trials of the inhibitory molecules showed promising results as single drug therapy or in combination with conventional chemotherapy. Further, multiple mutations associated with resistance to targeted therapy were identified, leading to treatment with second line drugs and consequent better prognosis. Further advancements of biotechnology with identification of genetic variation, multiple resistant mutations which help discovery of a cascade of genetic markers with deeper understanding of biology of disease that offers hopes towards identification of development of more efficient targeted therapy with reduced toxicity and resistance.

## Genetic Markers and Evolution of Targeted Therapy in Cancer

Advances in genomic technologies have resulted in remarkable progress in molecular diagnosis of cancer with identification of various unique genetic markers of pathogenic significance as targeted molecules. The targeted molecules comprise fusion genes, chimeric RNA, fusion/chimeric proteins, amplified genes, genes with point mutation, overexpressed/down regulated RNA and miRNA (Ali *et al.,* 2010; *Pavlovi et al.,* 2014; Shtivelman *et al.,* 1985,

**Key words:** Genetic markers, Targeted molecule, Targeted Therapy, Tyrosine kinase.
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Sjogren *et al.,* 1998). The genomic alterations have led to precise WHO classification of hematological malignancies resulting in differential diagnosis and stratification of patients for appropriate treatment protocols. The routine methods used in cancer include FISH, PCR, ARMS-PCR, RFLP, Real Time PCR, capillary electrophoresis, Sanger sequencing/pyrosequencing, microarrays for whole genome/ transcriptome/protein analysis, mRNA and methylotype analysis (Ku *et al.,* 2013; Sethi *et al.,* 2013; Staehler *et al.,* 2012).

Several target molecules in cancer are tyrosine kinases, as the tyrosine kinase signaling initiates molecular cascades leading to cell proliferation, differentiation, apoptosis, migration, invasion, and angiogenesis in the malignant tissues. Hence, identification and development of tyrosine kinase inhibitors as therapeutic agents has revolutionized cancer therapy (Sawyers, 2002). Epidermal growth factor receptor (EGFR) is the first receptor tyrosine kinase (RTK) played an important role in the identification of significance of tyrosine kinases in cancer (Carpenter *et al.,* 1978). The tyrosine kinases are primarily RTKs e. g. EGFRs (EGFR-1, EGFR-2, EGFR-3), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor (VEGF) receptor, and non-receptor tyrosine kinases (NRTK), e. g. SRC, ABL1, Janus kinase. The RTKs are activated by ligands, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) by binding to the extracellular domain of the receptors (Fig. 1).

The identification of the pathogenic molecules led to development of inhibitors as targeted drugs, impacting pharmacogenomics and personalized medicine. Targeted therapy directly interacts with pathognomic molecule, as against the cytotoxic drugs that primarily kill mitotic cells by interfering with cell cycle.

Targeted cancer drugs are generally monoclonal antibodies and small molecule inhibitors. Therapeutic monoclonal antibodies target specific antigens on the cell surface, such as transmembrane receptors, or extracellular growth factors, CD20, CD33, and CD52, present on leukemic and lymphoproliferative cells. Molecules associated with the immune mechanisms led to monoclonal antibodies – Rituximab, against CD20 (Table 1) in non-Hodgkin

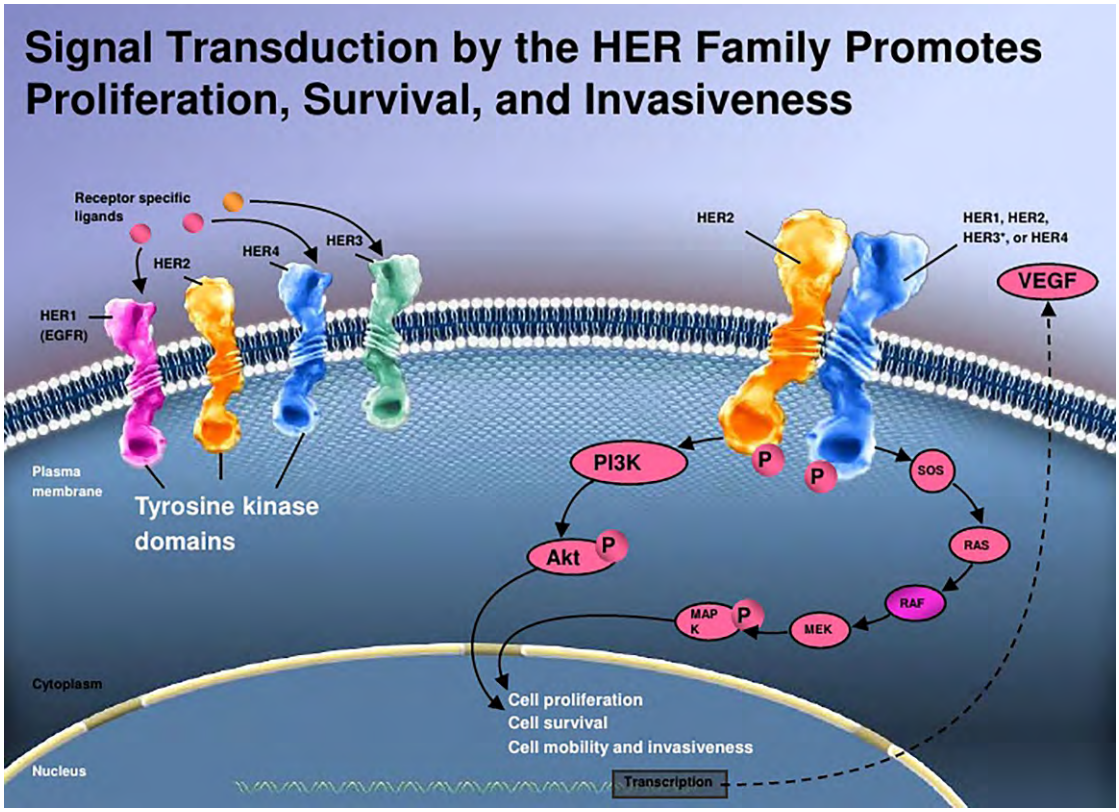


**Figure 1:** Activation of HER family receptors and signaling pathways (Adapted from: Hudis, 2007).

lymphoma (Silverman, 2007), and several monoclonal antibodies used in cancer treatment resulting in better prognosis (Fig. 1; Table 1). The monoclonal antibodies also target extracellular components of signaling pathways, including ligands and receptor binding domains blocking receptor signaling and downstream intracellular proteins involved in cellular proliferation, angiogenesis and invasion.

Small molecules inhibitors penetrate the cell membrane interacting with enzymatic activity of proteins, thereby blocking receptor signaling and interfering with downstream intracellular molecules (Fig. 2). Several growth factor receptors with intrinsic tyrosine kinase activity are constitutively active in cancers and inhibition of the kinases using small molecule inhibitors sensitizes the tumor cells to apoptosis.

RTKs are preferred key targets for anti-cancer drugs as aberrant activation of the RTKs usually result in downstream signaling with activation of pivotal cytoplasmic serine/threonine kinases (STKs). Small molecule cancer inhibitors targeting extracellular RTKs and cytoplasmic STKs are extensively studied

**Table1.** Molecular targets and targeted therapies in cancer (Abramson, 2015)

| Agent | Target (s) | Malignancy |
|---|---|---|
| Ado-trastuzumab emtansine(Kadcyla) | HER2 (ERBB2/neu) | Breast cancer (HER2+) |
| Lapatinib (Tykerb) | HER2 (ERBB2/neu), EGFR(HER1/ERBB1) | Breast cancer (HER2+) |
| Trastuzumab (Herceptin) | HER2 (ERBB2/neu) | Breast cancer (HER2+), Gastric cancer (HER2+) |
| Afatinib (Gilotrif) | EGFR(HER1/ERBB1), HER2(ERBB2/neu) | Non-small cell lung cancer (NSCLC)(with EGFR exon 19 deletions or exon 21 substitution |
| Ceritinib (Zykadia) | ALK | Non-small cell lung cancer |
| Crizotinib (Xalkori) | ALK, MET | Non-small cell lung cancer |
| Gefitinib (Iressa) | EGFR (HER1/ERBB1) | Non-small cell lung cancer with known prior benefit from Gefitinib |
| Ramucirumab (Cyramza) | VEGFR2 | Gastric cancer, adenocarcinoma, Non-small cell lung cancer |
| Axitinib (Inlyta) | KIT, PDGFRβ, VEGFR1/2/3 | Renal cell carcinoma ( RCC) |
| Bevacizumab (Avastin) | VEGF ligand | Cervical cancer, Colorectal cancer,Glioblastoma,NSCLC, Ovarian cancer, RCC |
| Pazopanib (Votrient) | VEGFR, PDGFR,KIT | Renal cell carcinoma |
| Temsirolimus (Torisel) | mTOR | Renal cell carcinoma |
| Panitumumab(Vectibix) | EGFR(HER1/ERBB1) | Colorectal cancer (KRAS wild type) |
| Cetuximab (Erbitux) | EGFR (HER1/ERBB1) | Colorectal cancer (KRAS wild type), Squamous cell cancer of head and neck |
| Everolimus (Afinitor) | mTOR | Pancreatic neuroendo tumor, RCC, Breast cancer (HR+, HER2-) |
| Erlotinib (Tarceva) | EGFR (HER1/ERBB1) | NSCLC, Pancreatic cancer |
| Bortezomib (Velcade) | Proteasome | Multiple myeloma, Mantle cell lymphoma |
| Carfilzomib (Kyprolis) | Proteasome | Multiple myeloma |
| Brentuximab vedotin (Adcetris) | CD30 | Hodgkin lymphoma, Anaplastic large cell lymphoma |
| Alemtuzumab(Campath) | CD52 | B-cell Chronic lymphocytic leukemia(CLL) |
| Dabrafenib (Tafinlar) | BRAF | Melanoma (with BRAF V600 mutation) |
| Ibritumomab tiuxetan (Zevalin) | CD20 | Non-Hodgkin's lymphoma |
| Idelalisib (Zydelig) | PI3Kδ | CLL, Follicular B-cell NHL, Small lymphocytic lymphoma |
| Rituximab (Rituxan, Mabthera) | CD20 | Non-Hodgkin's lymphoma,Chronic lymphocytic leukemia |
| Imatinib (Gleevec) | KIT, PDGFR, ABL | GI stromal tumor (KIT+),Hematologic malignancies including, Ph +ve ALL and CML |
| Nilotinib (Tasigna) | ABL1 | CML. |
| Ponatinib (Iclusig) | ABL1, FGFR1-3, FLT3, VEGFR2 | CML, ALL- Ph positive |
| Ruxolitinib (Jakafi) | JAK1/2 | Myelofibrosis |

(Arora *et al*., 2005)) Deregulated activation of RTKs results in increased cell growth and survival, and contributes to progression of cancer.

Targeted cancer drugs are designated as per the content of basic compound like monoclonal antibodies that end with "-mab", e.g., Rituximab, whereas small molecules end with the stem "-ib" indicating protein inhibitory action of targeted drug. For example, the small molecule STI-571 known as Imatinib (generic name) in which tinib indicated tyrosine kinase inhibitor (TKI). Drug with stem "-zom-" indicates proteasome inhibitors, e.g., Bortezomib. Small

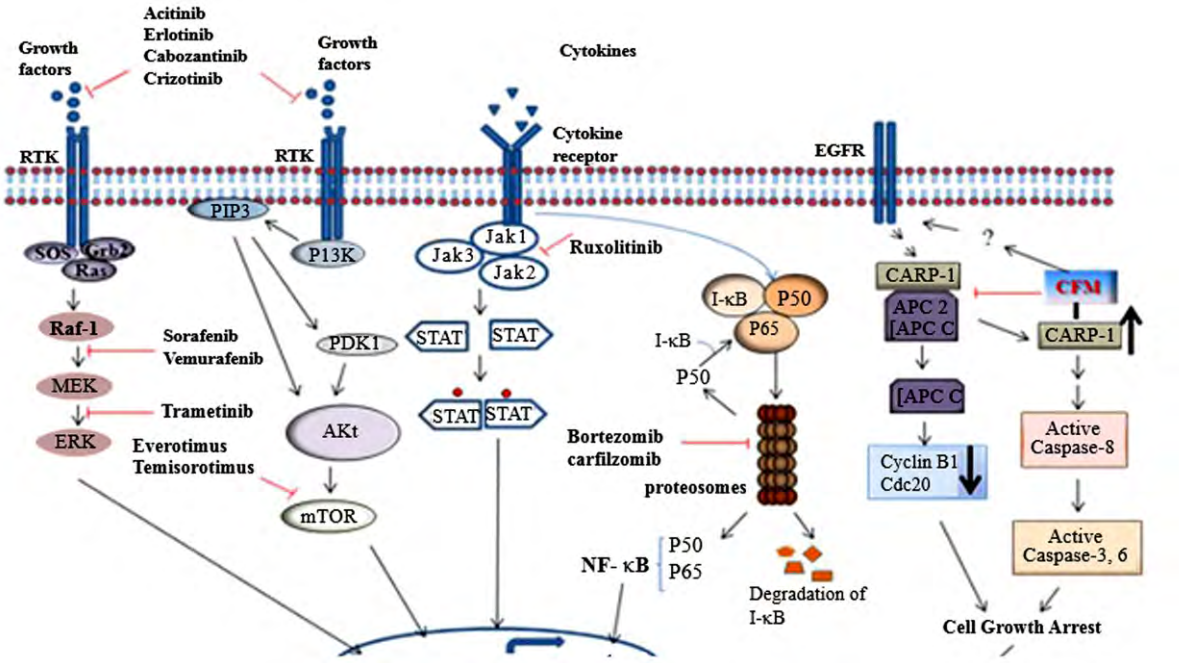


**Figure 2:** Schematic representation of activated cellular pathways in cancer and mechanism of small molecule inhibitors (Source: Lavanya *et al.*, 2014).

molecule inhibitors, tyrosine kinase inhibitors interrupt various intracellular signaling pathways of tyrosine kinases (Table 1).

## Tyrosine Kinase Deregulation and Targeted Therapy in Hematolymphoid Malignancies

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder associated with reciprocal translocation between chromosomes 9 (*BCR*) and 22 (*ABL*1) juxtaposing *BCR* sequences to *c-ABL*. c-ABL is a tyrosine kinase located at chromosome 9q34, resulting in constitutive production of fusion chimeric protein p210 with increased tyrosine kinase activity. The deregulated kinase activity usurps the physiologic functions of normal ABL enzyme by interacting with a variety of effecter proteins, resulting in deregulated cellular proliferation, decreased adherence of leukemic cells to the bone marrow stroma and a reduced apoptotic stimuli (Deininger *et al.,* 2000).

In acute lymphoblastic leukemia (ALL), TEL-ABL protein is constitutively phosphorylated due to reciprocal translocation t(9;12) (Hannemann *et al*., 1998). Chronic myelomonocytic leukemia (CMML) with t(5;12) produces TEL-PDGFRB fusion protein, leading to tyrosine kinase activation (Golub *et al.,* 1994). NPM1-ALK fusion product of

t(2;5) is constitutively activated in anaplastic large cell lymphoma (Shiota *et al.,* 1995).

Imatinib mesylate, a tyrosine kinase inhibitor in CML (Druker *et al.,* 2001), acts via competitive inhibition at the ATP-binding site of the BCR-ABL1protein, resulting in inhibition of phosphorylation of the downstream cascade of proteins in signal transduction pathways. Imatinib mesylate prevents BCR-ABL enzyme from permanent deactivation, thus inhibiting proliferation of leukemic cells and leading to apoptosis (Table 1) (Deshmukh *et al.,* 2005; Druker *et al.,* 2001). Imatinib mesylate efficiently inhibits BCR-ABL kinase, blocks platelet-derived growth factor receptor, and c-kit tyrosine kinase (Druker *et al.,* 2000). However, about 90 kinase domain mutations have been identified in *ABL1*, which prevents binding of the drug and thus induce resistance to the drug. Consequently, second generation tyrosine kinase inhibitors, Nilotinib, Dasatinib (Jabbour *et al.,* 2014; Kantarjian *et al.,* 2010) and Bosutinib (Khoury *et al.,* 2012) were developed to overcome resistance to Imatinib mesylate due to kinase domain mutations. Second generation TKIs overcome resistance of Imatinib. HoweverT3151, "gatekeeper" mutation, displays resistance to all second generation TKIs. Ponatinib, a third generation TKI, has overcome resistance due to kinase mutation T3151 (Jabbour *et al.,* 2014; O'Hare *et al.,* 2009) (Table 1).

In acute promyelocytic leukemia (APL), fusion gene *PML-RARA of* t(15;17) leads to a differentiation block in the abnormal promyelocyes. The targeted drug all-trans-retinoic acid (ATRA) leads to conformational change of PML-RARA protein followed by activation and regulation of RARA-responsive genes leading to differentiation of promyelocyes to granulocytes (Advani *et al*., 1999; Grignani *et al.,* 1998). The remission rates were significantly high in APL patients treated by ATRA. However, resistance to ATRA was observed in 25–30% of APL patients (Estey *et al.,* 2006), and arsenic trioxide (ATO) was found to be more efficient than ATRA as it induced apoptosis in addition to differentiation.

Besides, translocations, epigenetic silencing is an important genetic alteration leading abnormal expression of genes involved in cell cycle control and differentiation in AML. The replacement of cytosine by 5-aza-cytidine, a cytidine analogue, acts as a block to DNA methyl

transferases, causing demethylation of DNA and consequent differentiation (Egger *et al.,* 2004). Histone deacetylase (HDAC) inhibitors Vorinostat (Zolinza) and Panobinostat are additional agents for modulation of transcriptional repression of tumor suppressor proteins (Bolden *et al.,* 2006).

The two most prominent mechanisms in Myelodysplastic syndromes (MDS), DNA methylation and histone acetylation play a role in hematopoiesis. Methylation is focally increased around tumor suppressors and other mitogen inhibitors. DNA methyl transferases (DNMTs) play a role in increased methylation and hence a key target for treatment of MDS (Shih *et al.,* 2012). In high risk MDS, a number of genes associated with DNA repair, cell-cycle control, regulation of development, differentiation and apoptosis are hypermethylated in 70% of patients. The critical hypermethylated genes are *ALOX12, GSTM1, HIC1, FZD9, TET2* and *HS3ST2* (Jiang *et al.,* 2009). These hypermethylated genes are potential targets for demethylating agents. Patients with hypermethylated*TET2* showed better response rates (82%) on treatment with demethylating drug azacytidine than those with wild-type *TET2* (45%) (Itzykson *et al.,* 2011).

*JAK2* mutation has been reported in myloprolifertaive disorders Polycythemia Vera, Primary Myelofibrosis and Essential Thrombocythemia. *JAK2* encodes an on-receptor tyrosine kinase associated with signal relays for hemopoietic cell growth, development and differentiation (Neubauer *et al.,* 1998). Ruxolitinib, a JAK inhibitor showed promising results in patients with Myelofibrosis (Harrison *et al.,* 2012).

*BRAF* is a potent activator of MAP/ERK kinase pathway associated with regulation of cell cycle, differentiation and cell survival. *BRAF* mutations have been reported in solid cancers and hematopoietic cancers (Davies *et al.,* 2002; Holderfield *et al.,* 2014). The most common *BRAF* mutation is the V600E mutation (Holderfield *et al.,* 2014). Vemurafenib, a small molecule inhibitor showed anti-melanoma activity against the *BRAF* V600E mutant protein (Tsai *et al.,* 2008). Hematolymphoid malignancies including hairy cell leukemia and multiple myeloma with *BRAF* V600E mutation, showed favourable clinical response on treatment with Vemurafinib (Machnicki *et al.,* 2014) (Table 1).

Fms-like tyrosine kinase 3, CD135 (FLT3) a tyrosine kinase receptor is activated when bound by the FLT3 ligand (FL), subsequently promoting homo-dimerization. This switches tyrosine kinase activity of FLT3 followed by recruitment and phosphorylation of intracellular proteins SHC, GRB2, SHIP, CBL, CBLB-related protein domain, further leading to activation of MAP kinase, STAT and AKT/PI3 kinase signal transduction pathways. The proteins are transported to the nucleus regulating cellular proliferation, differentiation and apoptosis (Zhang *et al.,* 1999). *FLT3-ITD* (Internal tandem duplication) is a common mutation in 15–35% AML (Stirewalt *et al.,* 2006) and 5–10% MDS. *FLT3-ITD*and allelic variation in patients influences prognosis of AML patients (Meshinchi *et al.,* 2006). *FLT3*-TKD (Tyrosine kinase domain) mutation occurs in codon 835 (D835). Sorafenib, a tyrosine kinase inhibitor specifically targets the leukemic blasts in AML (Williams *et al.,* 2012) (Table 1).

Upregulation of JAK2 in AMLc ells results in resistance to*FLT3-TKI* inhibition (Ikezoe *et al.,* 2011). Second generation drug, Quizartinib (AC220) was potent in *FLT3-TKI* resistant cases due to upregulation of JAK2 (Cortes *et al.,* 2011). Pacritinib (SB 1518)is another potent JAK2/FLT3 inhibitor, in combination with Pracinostat (SB939), an oral HDAC inhibitor, showed synergy in inducing remission and better survival in the patients (Novotny-Diermayr *et al.,* 2012).

Nucleophosmin (NPM1) mutations result in overexpression of the phospho-protein in 27–35% of adult AML and 40–60% of adult AML with normal karyotype (Falini *et al.,* 2005). *NPM1* mutation occurs due to four base sequence TCTG duplication at position 956–959 in *NPM1* gene (Falini *et al.,* 2005). Inhibitors of NPM1 oligomerization such as NSC348884 increase apoptosis when exposed to the ATRA plus cytarabine combination (Balusu *et al.,* 2011).

CCAAT/enhancer binding protein alpha (*CEBPA)* protein is a key regulator of granulocytic differentiation (Rosenbauer *et al.,* 2007). *Hence, CEBPA* mutations induce proliferation and block differentiation of myeloid lineage. *CEBPA* mutation occurs due to N-terminal frame-shift mutations and secondly due to C-terminal in-frame insertions or deletions. *CEBPA* mutations frequently (70%) occur in AML patients exhibiting a normal karyotype. AML patients with a normal

karyotype and *CEBPA* mutation in the absence of *FLT3* show favorable prognosis (Green *et al.,* 2010).

C-KIT, a stem cell gene, encoding tyrosine kinase, demonstrated c-KIT mutations in AML patients with core binding factor rearrangement. Upon binding of the ligand stem cell factor, to c-kit, phosphorylation of several cytoplasmic proteins occur followed by activation of downstream MAP kinase, JAK/STAT, and PI-3 kinase pathways (Linnekin, 1999). Mutations in *c-KIT* receptor result in constitutive phosphorylation and activation of the receptor in absence of the ligand. Mutations in c-*KIT* and *FLT3* genes are associated with unfavorable prognosis in patients with t(8;21). In particular, patients with *c-KIT* mutation have been reported to have a higher incidence of relapse (80% versus 13. 5%) (Pascka *et al.,* 2004). *In vitro* studies have shown sensitivity to Imatinib for a mutation in exon 8 and exon 17. APcK110, with potent proapoptotic and antiproliferative activities has shown promising results in AML cell lines and primary samples (Faderl *et al.,* 2011).

BCL2, an anti-apoptotic protein, is overexpressed in hematological malignancies and is a possible molecule for targeted therapy. AML patients treated with Bcl-2 antisense oligonucleotide based therapy inhibit Bcl-2 over-expression, promote apoptosis and reduce drug resistance (Marcucci *et al.,* 2003).

**Targeted Therapy in Solid Tumors**

According to National Comprehensive Cancer Network (NCCN) guidelines, several molecular markers have been identified as targets for therapy in solid tumors. The molecular markers include *HER2 (ERBB2*) amplification in breast cancer, *K-RAS* and *BRAF* mutations in colorectal cancer, and *BRAF* v600 mutation in melanoma, *EGFR* mutation/*ALK*/rearrangement in non-small-cell-lung-cancer (NSCLC), and *c-KIT* in gastrointestinal stromal cancer. The following section discusses the markers in specific cancers.

**Molecular Markers and Targeted Therapy in Lung Cancer**

Lung cancer is the most common cancer in men globally with about 15% five year survival rates. Based upon various driver mutations, NSCLC is stratified based on the molecular lesions as NSCLC with *K-RAS* mutation, *EGFR* mutation, echinoderm microtubule-associated

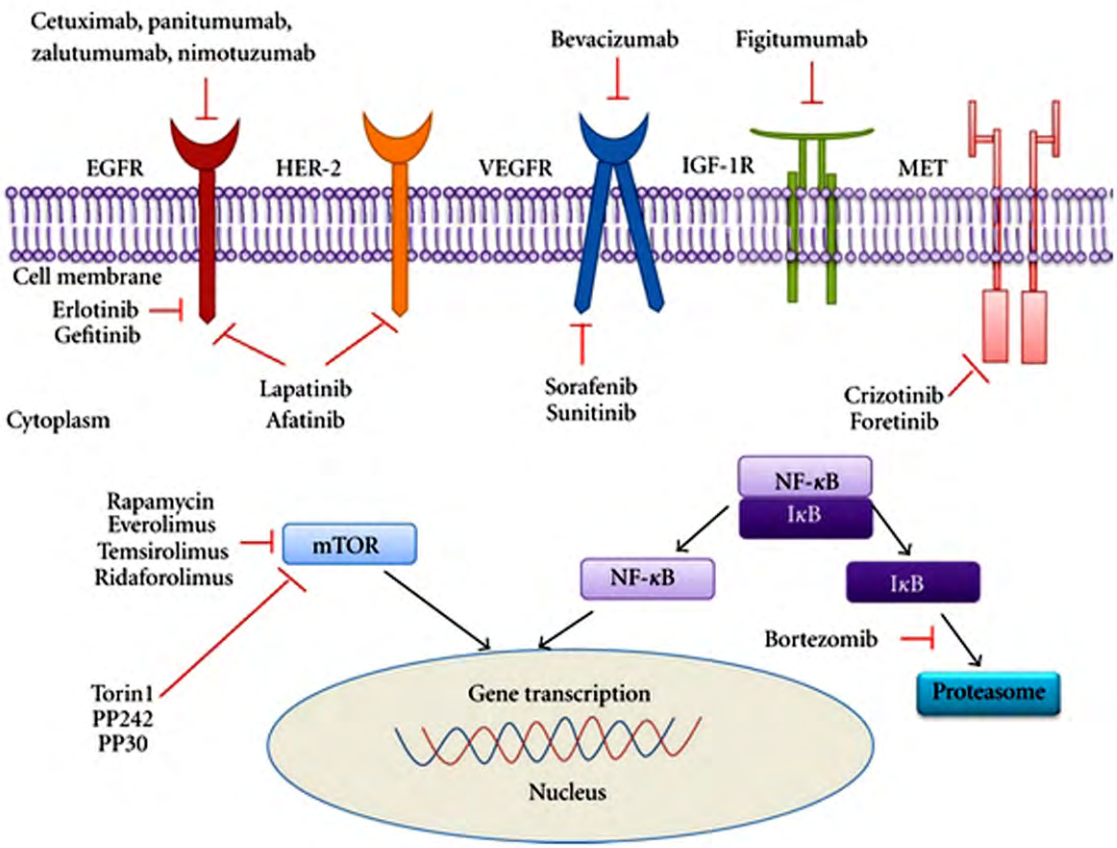


**Figure 3:** Mechanism of targeted therapies in patients with HER2 and EGFR, VEGFR and IGF-1R mutation (Source: Gao *et al*., 2012).

protein like 4-anaplastic lymphoma kinase (*EML4-ALK*) mutation, *herceptin 2 (HER2)*mutation, *v-raf* murine sarcoma (*BRAF*) mutation, mesenchymal epithelial transcription factor (*Met*) mutation, protein kinase B (*PKB/AKT1), phosphatidylinositide 3 kinase catalytic subunit (PI3KCA)* mutation (Pao *et al.,* 2011).

EGFR plays a critical role in cell proliferation, angiogenesis, and inhibition of apoptosis. *EGFR* mutation is reported in 10% of NSCLC in US, and 35% in Asian population (Pao *et al.,* 2011). The *EGFR* mutation is observed in less than 5% squamous cell cancer patients and 15–20% adenocarcinomas including females (never smokers) (Pao *et al.,* 2010). *EGFR* mutations are located in the kinase domain at exons 18–21 (Kosaka *et al.,* 2009). *EGFR* amplification has also been reported in NSCLC patients and associated with bad prognosis. Patients stratified as NSCLC with *EGFR* mutation are effectively treated with targeted therapy Erlotinib or Gefitinib targeted to the deregulated EGFR (Lazarus *et al.,* 2013) (Figs. 3 and 4) (Table 1). EGFR TKI,

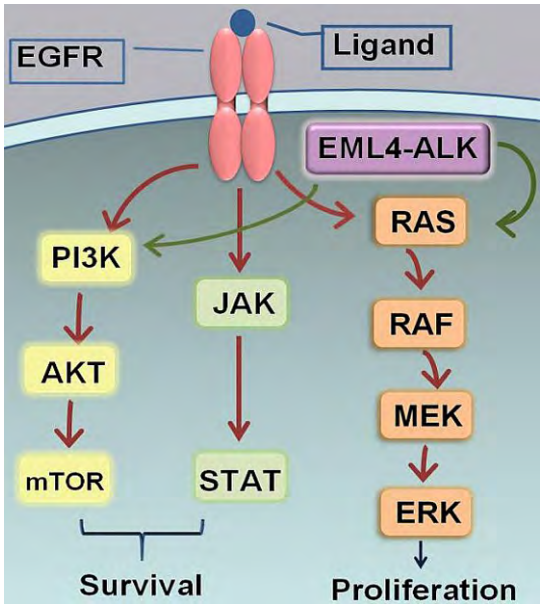


**Figure 4:** EGFR mutation, EML4-ALK translocation and Signaling (Source: Wu *et al*., 2012).

a small molecule inhibitor, therapy also shows better response to patients with *EGFR* amplification as compared with *EGFR* mutation.

An additional molecular lesion in lung adenocarcinomas is the point mutation in *K*-RAS gene, codon 12 or 13 (Knickelbein and Zang, 2015). *EGFR* mutation activates RAS signaling pathway downstream, hence patients with *K-RAS* mutation are resistant to EGFR TKI (Raponi, 2008).

ALK encodes a tyrosine kinase receptor normally expressed in selected neuronal cell types. *ALK-EML4* rearrangementtranslocation and balanced translocations retain ALK kinase domain with constitutive activation of tyrosine kinase, leading to transformation of cells (Soda, *et al.,* 2007) (Fig. 4). In Lung cancer, *ALK* rearrangement is detected by FISH with an *ALK* break-apart probe (Soda *et al.,* 2007). Lung cancer patients with *EML4-ALK* translocation show sensitivity to TKI inhibitor Crizotinib (Shaw *et al.,* 2011). However, resistance to the targeted therapy has been reported in patients with secondary mutations in *ALK* (Ettinger *et al.,* 2012; Sasaki *et al.,* 2011).

*FGFR1,* Fibroblast growth factor receptor 1 encodes a member of the FGFR tyrosine kinase family, with a critical role in cell development. *FGFR1 is* deregulated either by point mutation, translocation or amplification (Turner *et al.,* 2011). Preclinical trials with FGFR1 inhibitors have shown encouraging results in lung cancer (Weiss *et al.,* 2010). *FGFR1* amplifications are also observed in 20%in smokers with squamous cell sarcoma.

### *K-RAS* Mutations and Targeted Therapy in Colorectal Cancer

KRAS is a membrane bound GTPase, active in the GTP-bound form and inactive when GDP-bound. KRAS activity mediates a cascade of intracellular signaling events initiated by the ligand-receptor binding of RTKs, including EGFR (Downward *et al.,* 2003). EGFR

upon binding to its ligand is auto-phosphorylated creating a docking site for the adaptor protein growth factor receptor bound protein 2 (GRB2), resulting in activation of KRAS GTP, which further stimulates downstream signaling pathways, RAF/MEK and PI3K (and phosphoinositide-3 kinase)/AKT controlling cell growth and survival (Downward *et al.,* 2003) (Fig. 4). *K-RAS* mutations resulting in constitutive activation of RAS with expression of RAS proteins are reported in 20–25% of several human tumors including pancreatic cancer with *K-RAS* mutation in 90% (Downward *et al.,* 2003). The potent transforming mutations are detected in codons 12 (82% of *K-RAS* mutations) and 13 (17%) in exon 2 of the *K-RAS* gene (Wang *et al.,* 2010). *K-RAS* gene mutations predict outcome of treatment with anti-EGFR antibodies in advanced colorectal cancer (CRC).

Cetuximab, a human–mouse chimeric IgG1 monoclonal antibody, EGFR-targeted agent approved for the treatment of colorectal cancer (Jonker *et al.,* 2007) (Fig. 3), and Panitumumab are commonly used in CRC therapy (Heinemann *et al.,* 2013). Bevacizumab (Avastin), Ramucirumab (Cyramza), and Ziv-aflibercept (Zaltrap) are drugs used for colon cancer that target VEGF (Douillard *et al*., 2014). These drugs are combined with chemotherapy to treat advanced colon cancer (Table 1). Farnesyl transferase inhibitors (FTIs) are small molecule inhibitors that selectively inhibit farnesylation of a number of intracellular substrate proteins such as RAS, an additional approach to target *K-RAS* mutations (Gysin *et al*., 2013). However, a comprehensive understanding of RAS mediated signal transduction feedback loops, tumor heterogeneity and mechanisms of downstream targets of *K-RAS* gene on CRC is needed for optimal use of the monoclonal antibodies, small molecular inhibitors to *K-RAS* abberations.

### *HER2* Marker and Targeted Therapy in Breast Cancer

*HER2* amplification has been observed in 20% invasive breast carcinomas, and is a poor prognostic marker with an increased risk of disease progression, recurrence of disease with poor survival (Andrulis *et al.,* 1998). FISH is an efficient tool for detection of *HER2* amplification. *HER2* encodes a transmembrane tyrosine kinase receptor in the EGFR family. *HER2* stimulates growth factor signaling

pathways such as PI3K–AKT–mTOR pathway (Fig. 1). Trastuzumab (Herceptin), a humanized, recombinant monoclonal antibody that binds to the extracellular domain of HER2 is an efficient targeted therapy (Vogel *et al.,* 2002) (Fig. 3). Trastuzumab selectively blocks ligand independent HER2–HER3 dimerization and proteolytic cleavage of the extracellular domain of HER2 resulting in downregulation of PI3K pathway signaling and downstream cell cycle protein cyclin D1 (Junttila *et al.,* 2009). Herceptin resistance is seen in several breast cancer patients with mutational activation of P13K pathway through loss of *PTEN*, indicating PI3K-based treatment options. Lapatinib, an ATP-competitive inhibitor of HER2 and EGFR tyrosine kinases, have shown efficacy in Trastuzumab resistant patients (Konecny *et al.,* 2006). Pertuzumab monoclonal antibody binding to a distinct epitope on the extracellular domain of HER2 blocks ligand induced dimerization of HER2 and HER3 (Junttila *et al.,* 2009) (Table 1).

BRAF V600E mutation occurs in 60% melanoma patients. The mutation constitutively activates mitogen activated protein kinase (MAPK) pathway, promoting cell proliferation and preventing apoptosis (Gray-Schopfer *et al.,* 2007). Hence, *BRAF* V600E mutation is considered as a promising therapeutic target in metastatic melanoma. Vemurafenib treatment in patients with *BRAF* V600mutant metastatic melanoma indicated that inhibition of MAPK pathway promoted cell proliferation and prevented apoptosis (Flaherty *et al.,* 2010). Vemurafenib induces clinical responses in 50% patients with *BRAF* V600 mutant metastatic melanoma. Vemurafenib and Dabrafenib are effective targeted drugs for melanomas with *BRAF* V600Emutation (Kim *et al.,* 2014) (Table 1).

## CONCLUSION

A continuous research efforts by various genomic technologies made remarkable progress in the discovery of genetic markers which have diagnostic as well as prognostic significance in hemato-lymphoid malignancies and solid tumors as well. Driver mutations and their mechanism of actions disclosed role of various oncogenic pathways that contributed significantly in the development of effective inhibitor molecules/proteins as targeted therapy.

Clinical trials of the inhibitor molecules have shown promising results in comparison with traditional cytotoxic chemotherapy. Further advancement in genomics is expected to identify cascade of genetic markers help understanding biology of disease that offers hopes towards development of more efficient targeted therapy with reduced toxicity and resistance.

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Review

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# Cancer gene therapy: Prospects of using human sodium iodide symporter gene in non-thyroidal cancer

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Gene therapy is one of the promising therapeutic strategies evolved rapidly in the frontier of translational biology in cancer. To overcome the off target effect of conventional cancer therapies it is the most flourishing approach in present epoch. Various researches in this context are ongoing to eradicate devastating cancer cells with minimal or no side effects. Of the various gene therapy protocols developed, a set of genes called suicide genes, are being actively pursued as potential strategy. Briefly, this strategy involves tumor targeted delivery of a therapy/reporter gene to convert a systematically administered pro-drug into a cytotoxic drug which in turn induces tumor cell death. Additionally, advancement in small animal imaging modalities facilitates real-time monitoring of the delivered transgene by using appropriate imaging probe developed against the transgene. Non-invasive monitoring helps to realize precise transgene delivery and also aid to understand therapy response. In this background, we have reviewed potential suicide genes frequently explored for cancer treatment, which supports both diagnostic and therapeutic applications with special emphasis on sodium iodide symporter (NIS). Apart from its natural expression in thyroid, NIS protein expression has raised the possibility of using radioiodide therapy and diagnosis in few non-thyroidal cancers as well. In this review, we also covered various challenges to get NIS gene therapeutics from bench to bedside in various non-thyroidal cancers.

## Gene Therapy for Cancer

With rapid advances in cellular and molecular understanding in the genome era, gene therapy holds great potential in treating various human diseases including cancer. The science behind gene therapy relies on introducing genes to cure or retard the progression of the disease. Theoretically, by introducing necessary modifications for the mutated part(s) of a gene or by replacing the defective gene as a whole, one can potentially cure or retard the severity of a disease caused by the effect of a single gene. However, in reality, cancer is mostly considered as a multi-gene disorder (LaDuca *et al*., 2014). Though many cancers have a genetic predisposition, a majority of them have

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acquired mutations and chromosomal abnormalities. As the disease progress, the cells become less differentiated and more heterogeneous with respect to the mutations they carry. The range of genes involved or the mutations they carry have grown into a long-winded task for gene therapy field to solve. The ability to image the location(s), magnitude, and real-time variation of therapeutic gene expression has become a key force in the rapid evolution of gene therapy. Another concern in gene therapy application is the need to achieve controlled and effective gene expression in the target cells, with minimal or no off-target effect in neighboring normal tissue locations. To address this issue, *ex vivo* strategies help to ensure that gene transfer is limited to cells of a particular organ. For example, gene transfer into bone marrow cells provides a means to introduce genes selectively into various types of blood cells, including hematopoietic stem cells (Pan, 2009). However, ability of direct gene transfer to the appropriate cells via systemic delivery of a vector is often complicated, but appreciably enhances gene therapy approaches.

Coming to gene therapy of cancer, diverse applications reported in literatures can be broadly categorized into five subgroups based on their mechanism of actions: (i) suicide gene therapy which uses introduction of drug-sensitive genes for selective induction of cellular sensitivity to a prodrug, (ii) protection of sensitive tissues like bone marrow from otherwise toxic doses of a cancer drug by use of multidrug resistance genes, (iii) replacement of lost or loss-of-function tumor suppressor genes, (iv) compensating down-regulated oncogene or gain-of function oncogenic mutation, and (v) insertion of a cytokine gene into tumor cells *ex vivo*. In this review, however, we want to focus on the first approach, where the gene or transgene signature can be utilized for killing the same cells where it is being expressed. We will continue our discussion analyzing gene therapy applications using sodium iodide symporter (NIS) in various non-thyroidal cancers.

**Suicide Gene Therapy**

Eventually the need of a cancer therapy is for complete remission of the cancer cells causing minimal damage to the surrounding normal tissues. In this regard the most promising approach is the targeted suicide gene therapy. With recent

advances in vector design, improvements in transgene (a new or altered gene that is being introduced) and prodrug activation strategies, suicide gene therapy is being applied to a wide variety of cancers (Zarogoulidis *et al*., 2013). Till date various prokaryotic or eukaryotic genes have been tested as suicide gene therapy candidate, several of them also support diagnostic imaging to identify *in vivo* localization of the gene in action. For a particular therapeutic gene, diagnosis was done mostly by using gamma-ray emitting radionuclide probes while beta-ray emitting radionuclide probes are used for cancer cell diminution. In order to understand the consequences of the delivered transgene, non-invasive and real-time monitoring by using appropriate imaging modality is crucial. In this context reporter gene that supports radionuclide-based imaging approach gains attention as these imaging procedures are clinically relevant. With improved optical imaging instrumentations, the radionuclide based imaging applications has expanded scope now. Based on the phenomenon known as Cerenkov luminescence, emits visible luminescence photons when the charged radioactive particles travel through a dielectric media (such as tissue) at a speed higher than the speed of light, can be captured in real time to understand the tissue bio-distribution of radiotracers (reviewed in Thorek *et al*., 2012, Tanha *et al*., 2015). There are at least three different types of interactions between the reporter proteins with their probes, which include an enzyme-based (e.g. Thymidine kinase and Cytosine deaminase), receptor-based (e.g. Somatostatin receptor) and transporter-based (e.g. NIS), used frequently for cancer gene therapy. We will now discuss ongoing research efforts utilizing these genes.

***Herpes Simplex Virus type 1 Thymidine kinase (HSV-1 TK)***

Among different suicide genes HSV-1 TK is the most frequently studied classical suicide gene target which converts non-toxic prodrug into a toxic drug. This viral TK phosphorylates various nucleoside analogues like aciclovir, ganciclovir, penciclovir much more efficiently than its mammalian homolog. Thus, mechanistically these cell-permeable substrates first get monophosphorylated by HSV-1 TK and subsequently phosphorylations are carried out by host kinase to generate the triphosphate form, which (deoxy-thymidine triphosphate) is basically a purine analog that inhibits DNA polymerase and therefore creates

toxicity to cause cell death eventually. Being highly proliferative in nature, cancer cells actively synthesize DNA, so the purine analog competes with guanosine triphosphates (GTPs) and get incorporated into the nascent DNA chain. As a result the nuclear and mitochondrial DNA synthesis terminates and cells are forced towards apoptosis. However, in transgenic mice the use of HSV-1 TK for tissue specific sensitization through ganciclovir treatment showed limitations. Due to high nuclear localization, the enzyme creates spermatozoal toxicity which in turn renders the male transgenic mice sterile (Cohen *et al*., 1998). In another study investigators have revealed that due to the presence of putative cryptic testis-specific promoter within the coding sequence, the HSV-1 TK gene exhibited such outcome (Salomon *et al*., 1995). Therefore to address this issue various studies have been carried out by generating different mutated version of HSV-1 TK with improved enzymatic activity and varying nuclear clearance (Ponomarev *et al*., 2003). Among those HSV-1 sr39TK (also termed as HSV-1 tTK) was the most successful mutant (Black *et al*., 2001) used recurrently for radionuclide imaging as well as suicide gene therapy purpose. Imaging of HSV-1 tTK using $^{18}$F-9-(4-[18F] fluoro-3-hydroxymethylbutyl) guanine ($^{18}$F-FHBG) and $^{18}$F- or $^{124}$I-2′-deoxy-2′-fluoro-5-iodo-1-[β]-D-arabino-furanosyluracil ($^{18}$F-FIAU and $^{124}$I-FIAU) positron emission tomography (PET) turned it into a imaging reporter for clinical use. Later successful combining strategies by fusing HSV-1 sr39TK to other reporters made it suitable for multimode imaging (De *et al*., 2003; Ray *et al*., 2003; Ray *et al*., 2004; Ruggiero *et al*., 2010; Serganova *et al*., 2008). In another study, a triple fusion gene construct using NIS, HSV1-sr39tk, and EGFP was developed and demonstrated its use as a suicide therapy agent in hepatocellular carcinoma (Lee *et al*., 2010). However majority of such fusion gene using HSV-1 TK are used as imaging reporter rather than suicide therapy purpose. Another therapeutic study noteworthy here is the first-in-man use of engineered T-cells with HSV-1 TK-truncated CD34 fusion. This work highlights the suitability of tCD34 as a GMP compliant selection marker and demonstrates the feasibility, safety and immunological potential of HSVTK-tCD34 suicide gene modified donor T cells (Zhan *et al*., 2013).

***Cytosine deaminase (CD)***

Cytosine deaminase protein is mainly synthesized in some bacteria and fungi, which deaminates cytosine to uracil. CD can also converts a non-toxic compound 5-fluorocytosine (5-FC) into the toxic compound 5-fluorouracil (5-FU) (Ramnaraine *et al*., 2003). It exerts toxic effect by replacing for uracil in cellular RNA and therefore interferes with DNA and protein synthesis. Basically the absence of CD in mammalian tissues allows its use as a drug for suicide gene therapy for various cancer treatments. In an *in vitro* study genetically engineered stem cells to produce CD converts non-toxic 5-FC to a cytotoxic agent 5-FU and after migrating towards tumor site exhibited reduction in tumor growth (Kim *et al*., 2010). Deamination of 5-FC prodrug produces two toxic metabolites such as 5-fluorodeoxyuridine monophosphate (FdUMP) and 5-fluorouridine triphosphate (FURTP). FdUMP being a potent inhibitor of thymidylate synthetase, an enzyme required for DNA synthesis, inhibits DNA synthesis and endorses apoptosis in tumor cells (Chen *et al*., 2007; Myers, 1981). In the first attempt suicide gene therapy using CD was demonstrated significant tumor reduction in rat glioma cells using 5-FC (Nishiyama *et al*., 1985). Several other studies have also demonstrated positive effect of CD/5-FC as an effective anti-tumorigenic system for therapy in different other cancers (Li *et al*., 2003; Lv *et al*., 2009; Yang *et al*., 2015). Improved tumor regression was obtained when radiation was combined with adenoviral mediated delivery of a mutated CD gene (Ad bCD-D314A) in pancreatic cancer (Kaliberova *et al*., 2008).

***Nitro-reductase (NTR)***

Another promising, but relatively less used, prodrug activation enzyme used in cancer therapy is nitro-reductase (NTR). NTR is a flavoprotein synthesized by *Escherichia coli*. One of its substrates is CB1954 (5-[aziridin-1-yl]-2, 4-dinitro-benzamide), which reacts with cellular thioesters and get converted into a potent DNA cross-linking agent by NTR resulting in inhibition of DNA synthesis. Therefore viral mediated delivery of NTR in tumor cells get sensitized upon administration of CB1954, demonstrated the basis of cancer gene therapy (Searle *et al*., 2004). An *in vitro* study showed that upon CD1954 treatment, NTR expressing clones (retro virus mediated delivery) of pancreatic and colorectal cancer cell lines

became 500 and 50 fold more sensitive than the parental cell line respectively (Green *et al*., 1997). In preclinical tumor xenograft model, tumor regression using NTR/CD1954 was also reported (McNeish *et al*., 1998; Weedon *et al*., 2000).

***Somatostatin receptor (SSTR)***

Somatostatin (SST) is a peptide hormone involved in various biological processes in normal human tissues. When SST interacts with SSTRs, it exhibits strong anti-proliferative effect in normal and tumor cells. SSTRs are consisting of G-protein coupled receptor subtypes (1-5), differentially expressed in various tumor types. Among these subtypes SSTR1 and SSTR2 has been reported to have pivotal role in anti-proliferative effect. However, SSTR2 is majorly studied in gene therapy for cancer treatment (Schaer *et al*., 1997). In most of the prostate cancer cases SSTR2 expression is found to be inactivated, so when the full length cDNA of SSTR2 was introduced using non-viral gene delivery system in the PC-3 cells, marked anti-angiogenic effect was reported (Kumar *et al.*, 2004). An *in vitro* study in pancreatic carcinoma also showed significant inhibition in cell proliferation upon delivery of an adenoviral mediated MUC1-promoter expressing SSTR2, although there was no AdMUC1-SSTR2-induced apoptosis (Chen *et al.*, 2005). *In vivo* study also showed impairment of tumor progression upon delivery of SSTR2 gene in pancreatic cancer (Carrere *et al.*, 2005). Interestingly neuro-endocrine tumors (NETs) have natural over expression of SSTRs, so detection and treatment of NETs become easier by using SSTRs. $^{68}$Gallium ($^{68}$Ga) labeled somatostatin analogue such as 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetra-acetic acid (DOTA) was used to image NETs through PET/CT which was found to be more efficient than the conventional SSTR scintigraphy (Gabriel *et al.*, 2007; Sollini *et al.*, 2014). Moreover, other somatostatin analogues like $^{177}$lutetium ($^{177}$Lu) or $^{90}$yttrium ($^{99}$Y) are also found to have potential use in therapy for NET patients (Kam *et al*., 2012; Sowa-Staszczak *et al*., 2011).

***Sodium iodide symporter (NIS)***

Human NIS expression in various cancers like thyroidal and several non-thyroidal malignancies allow its worldwide use for theranostic application. Being a member of the solute carrier transporter (SLC5A5),

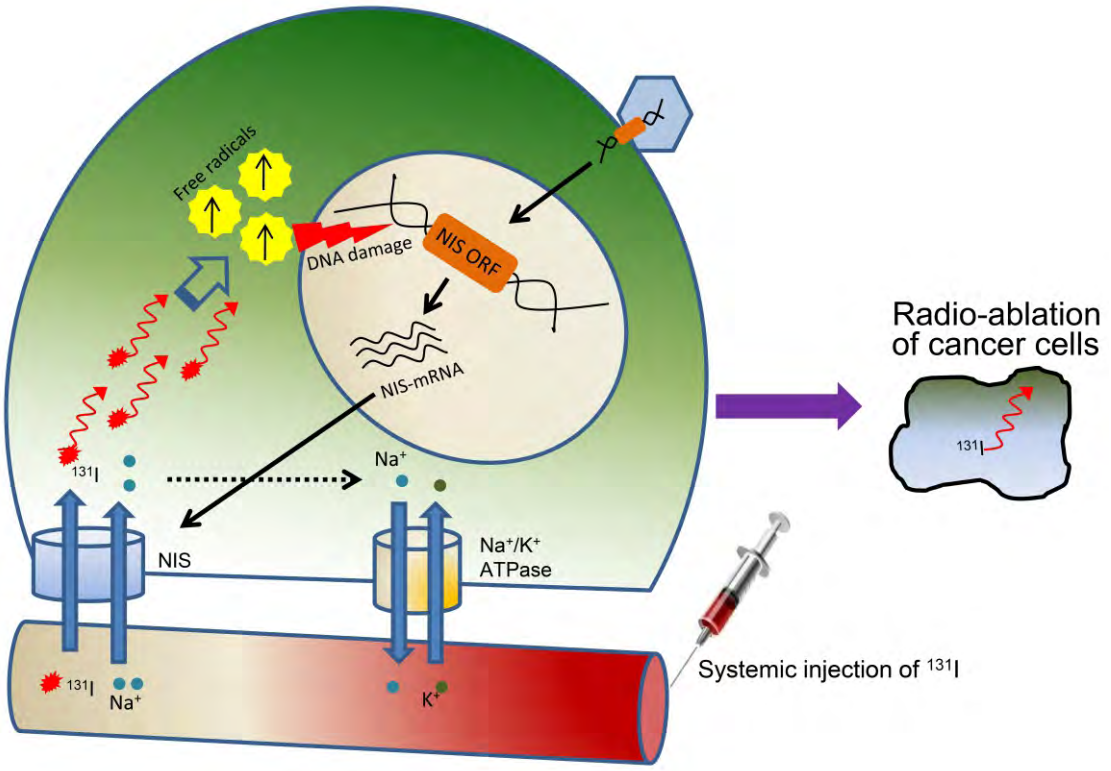


**Figure 1:** Mechanistic representation of NIS mediated gene therapy using $I^{131}$ administration. Being a $Na^+/I^-$ pump protein, NIS imports 2 $Na^+$ and one $I^-$ across the cell membrane where it expresses. Thus after achieving functional NIS expression administered $I^{131}$ permits selective ablation of neoplastic cells.

NIS is an intrinsic plasma membrane glycoprotein that mediates active iodide transport in thyroid follicular cells. NIS mediated iodide transport is also seen in extra-thyroidal tissues such as salivary gland, gastric mucosa and lactating mammary tissue. The natural function of this protein allows inward iodine transport to the cells providing the opportunity to use it for radioiodine mediated diagnosis and therapy (Fig. 1). Among various radionuclides $^{123}I$, $^{125}I$, $^{99m}TcO4$ are mainly used for SPECT (single photon emission computed tomography) and $^{124}I$ for PET based diagnostic imaging application while β-emitting $^{131}I$ is used for therapeutic application. However, measurement of therapeutic efficacy through imaging is often complicated as that will require delivery of two different radiotracers. Therefore, use of a surrogate marker (e.g. $^{18}F$-FDG PET scan) or an independent reporter based imaging (e.g. bioluminescence/fluorescence imaging) to monitor therapy effect can help circumventing the issue (Fig. 2A). For experimental and preclinical imaging applications, of course Cerenkov

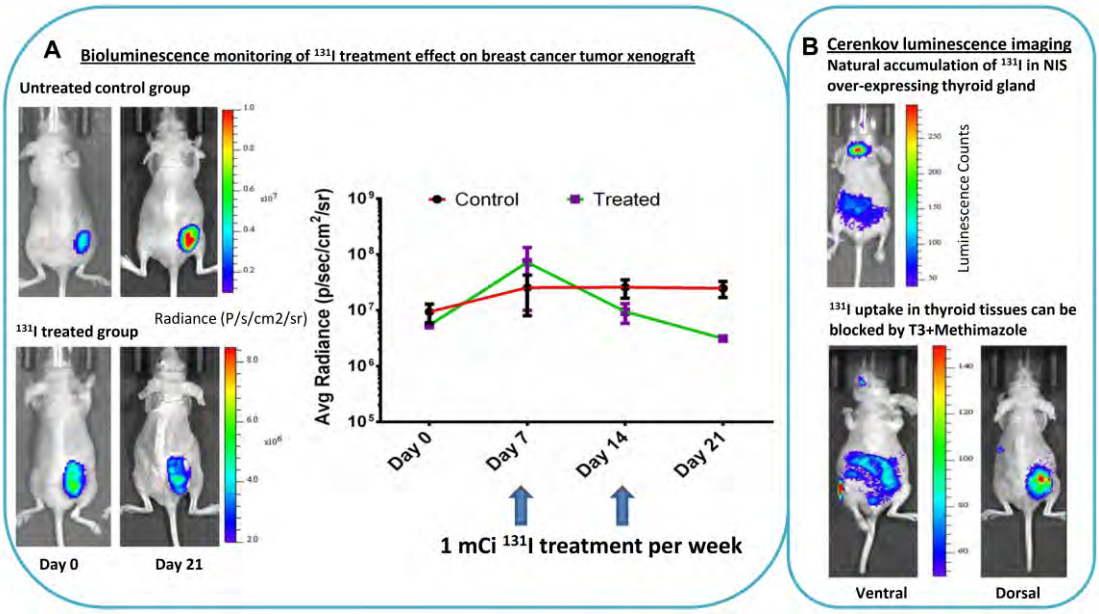


**Figure 2:** Optical luminescence imaging of breast cancer xenograft in mice undergoing $^{131}$I treatment. A. Bioluminescence imaging to evaluate $^{131}$I therapy in mice. These sets of mice were implanted with Zr75-1 breast cancer cells co-expressing human NIS and Firefly luciferase reporter gene. After a good size of tumor develops, mice in the treatment group received weekly dose of 1 milliCurie/mouse $^{131}$I on day 7 and 14 as indicated and monitored for attenuation in luciferase signal by injecting D-luciferin substrate. In comparison to the untreated control group, treated group shows significant diminution in tumor luciferase signal indicating NIS expressing tumor cell death. B. $^{131}$I being -emitter generates Cerenkov luminescence signal. This signal can be monitored using optical CCD camera. Representative mouse image on top shows natural accumulation of $^{131}$I in thyroid gland 24 hours after 1 milliCurie radioiodine was injected intra-peritoneal. Bottom image shows ventral and dorsal view of a tumor bearing mouse in which breast cancer xenograft was placed dorsally on the right flank. This mouse received 14 days pre-treatment with triiodothyroxin (T3) and methimazole (an iodine organification inhibitor) daily, showing possibility of thyroid blocking without affecting the radioiodine uptake in breast tumor xenograft.

luminescence imaging (Thorek *et al.*, 2012; Xu *et al.*, 2012) from most of these particulate emitters (except for $^{99m}$TcO4) supported by optical imaging modalities can be exploited (Fig. 2B). The ability of various radioisotopes producing Cerenkov radiation definitely add advantages such as low scan time requirement providing higher throughput and quantitative measurement of radioisotope distribution in the body, however the modality also suffers from issues such as lack of absolute quantitative ability and significant signal attenuation with greater tissue depths.

Gene therapy applications using NIS can be broadly categorized into two streams, transgene-mediated and endogenous and we will now discuss various cancer application aspects in detail in the following sections.

## Sodium Iodide Symporter as a Transgene Target for Cancer Treatment

Since, human NIS protein functions in a

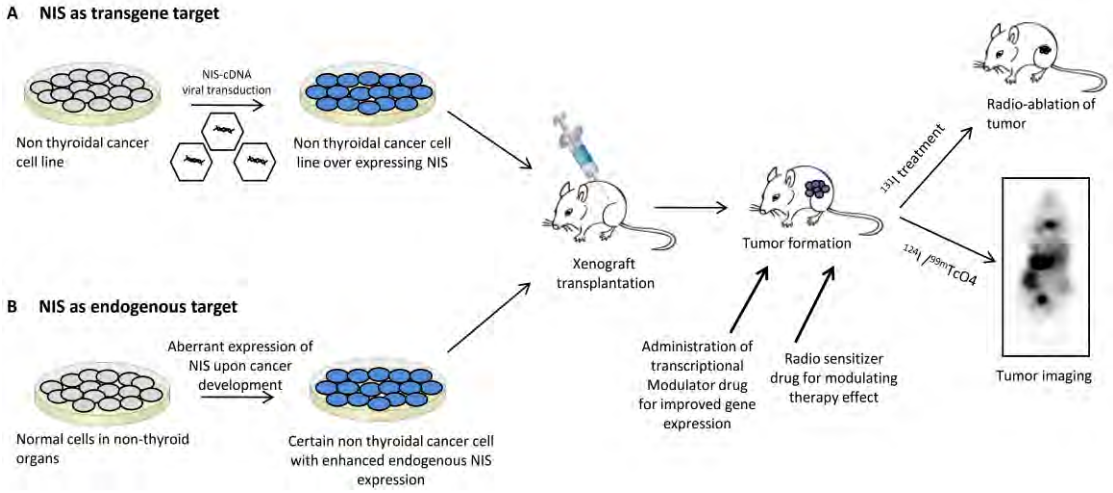


**Figure 3:** Schematic explaining various preclinical experimental set up for testing NIS gene mediated diagnosis and therapy in non-thyroidal cancers. A. NIS as transgene target is generally achieved by viral transduction methods in cancer cells. Such NIS over-expressing cells can be used for xenograft transplantation. Tumor formation can be visualized through appropriate imaging modality by administering $^{124}I$ / $^{99m}TcO4$. Treatment with $^{131}I$ leads to tumor ablation. B. NIS as endogenous target is often recorded in certain cancer types. Various pharmacological modulators can be tested to improve endogenous NIS expression and/or function in these cancer cells *in vitro* or *in vivo* tumor xenograft. Radio-sensitization of tumor could further improve treatment efficacy though $^{131}I$ treatment.

very limited number of tissues in human body (primarily in thyroid, salivary gland, gastric mucosa and lactating breast), it is an attractive target to treat cancer in tissue types where NIS has no/aberrant expression. Among various non-thyroidal cancers NIS (isolated from both rat and human) transgene mediated targeted radioiodine therapy was studied primarily in prostate, colon, ovarian and breast cancers (BCs). Adenovirus and retrovirus mediated NIS gene transfer has been attempted specifically to these tumor cells and in some cases there *in vivo* efficacy was also tested (Fig. 3A). NIS gene delivery in prostate cancer has been thoroughly explored since last decades. The first attempt of an *in vivo* adenovirus mediated hNIS (human NIS) delivery was done by Spitzweg et al. in non-thyroidal tumor xenograft. Significant NIS gene expression in prostate cancer xenograft was obtained followed by average reduction of tumor volume upon treatment with therapeutic $^{131}I$ (Spitzweg *et al.*, 2001). Later adenovirus mediated delivery of rat NIS (Ad-rNIS) was also reported in human prostate cancer tumor xenograft. The study also showed inhibition of tumor growth after intratumoral injection of Ad-rNIS followed by $^{131}I$ treatment (Spitzweg *et al.*, 2001). Therefore it is quite evident that rNIS has an equal potential compared to hNIS in suicide gene therapy for cancer

treatment. Further investigation has revealed that virus mediated NIS gene expression could be up-regulated through external beam radiation and DNA damage repair inhibitors (Hingorani *et al.*, 2008a; Hingorani *et al.*, 2008b). Additionally administration of radiosensitizing drugs further enhance therapeutic potential of NIS mediated $^{131}$I in colorectal and head and neck cancer (Hingorani *et al.*, 2010). Baculovirus mediated NIS gene delivery in colon cancer cell line also showed efficient cell death through $^{131}$I therapy (Yin *et al.*, 2010). In an *in vitro* study using ovarian cancer cell transduced with Ad/CMV/NIS and Ad/MUC1/NIS, 12- and 5-fold higher iodide uptake was reported compared to the parental cell line respectively. The same group has also shown reduction of ovarian tumor xenograft following $^{131}$I treatment upon CMV/NIS infection of the tumors (Dwyer *et al*., 2006).

On the other hand, in BC, aberrant over-expression of NIS is already reported; however the level of expression is often not sufficient for any therapeutic application. So, to achieve effective radio-iodine therapy, investigators adopted strategy to selectively transfer NIS gene in breast cancer cell types. In one of such studies engineered MCF7 cell line over expressing NIS and firefly luciferase was used to create mice xenograft and tested the effect of $^{131}$I through bioluminescent imaging. The pattern of tumor regression upon $^{131}$I administration in NIS expressing BC xenograft was also shown (Ghosh *et al*., 2006). Another study has generated a conditional adenovirus vector containing MUC1 promoter driven E1a gene and a transcriptional cassette RSV promoter-hNIS in the E3 region that can replicate only in the MUC1 positive cells. Therefore, the engineered virus particles were transduced into MUC1 positive T47D breast cancer cell line showing improved radioiodine uptake (Trujillo *et al.*, 2009). Interestingly, virus mediated NIS transgene expression was shown to be further induced by treating cancer cells with retinoic acid (RA) which also showed subsequent enhancement in radioiodine uptake in MCF7 cell lines (Lim *et al.*, 2007). Further, non-replicative adenovirus was used for targeted NIS gene delivery by using promoters of human telomerase subunits RNA (hTR) and human telomerase reverse transcriptase (hTERT), which are active only in cancer cells (Riesco-Eizaguirre *et al.*, 2011).

***Overcoming challenges of NIS gene delivery***

Although viral mediated NIS gene delivery is mostly attempted but this approach poses some risks for human application. The virus particles having their natural tropism towards certain cell types may have a natural tendency to infect and integrate and/ or re-combine within the genome. For example, in an initial clinical trial for NIS gene therapy in X linked severe combined immuno-deficiency, an incidence of leukemia was reported due to unexpected integration of the viral DNA in the host genome (Kogai *et al*., 2012). Therefore to avoid such unwanted DNA integration, all experimental NIS gene therapies are conducted with replication deficient viral vectors (Boland *et al*., 2000; Hutzen *et al*., 2012; Kim *et al*., 2007). However, immunogenic reactions by the host, mutagenic integration (retroviral and lentiviral vectors), inflammatory toxicity (adenoviral vectors), and large scale production of the viral particles (adeno-associated vectors) are major limitations for such applications (Duarte *et al.*, 2012; Witlox *et al.*, 2007). So, current attempts are oriented towards non-viral vectors as vehicles for gene/drug delivery. Cationic lipids, polymers, peptides and nano-particles have commonly been used for DNA delivery into the cells in this regard (Chen *et al*., 2015; Duarte *et al*., 2012; Fan *et al*., 2015; ). Unlike viral vectors, targeting of non-viral vectors is a major concern and that could be improved by conjugating non-viral vectors with ligands which bind to specific receptors or antigens expressed on the cancer cells (Ogris *et al*., 2002).

**Sodium Iodide Symporter as an Endogenous Target for Cancer Therapy**

Apart from introducing NIS gene in various cancer cells, ongoing active research has also exploited therapeutic potential of the gene where a specific cancer type is associated with aberrant over-expression of this gene. In such cases, since NIS protein is already present in the tumor cells, the burden of designing a vector for delivering the gene in the target cell is eliminated and thus can be spontaneously utilized for therapeutic intervention using radioiodine. Here, we have discussed various studies which have attempted ways to improve endogenous NIS expression and function for optimal therapeutic benefit (Fig. 3B). NIS is a true theranostic molecule which supports non-

invasive *in vivo* imaging using different form of radioactive iodine (PET, SPECT radiotracers) to judge sufficient iodine accumulation inside the tumor, which in turn indicates therapeutic success. Endogenous NIS gene therapy applications are primarily aimed at breast cancer. Apart from BC, modulation strategy to alter NIS aberrant expression was so far known from hepatocellular and testicular cancers (Guerrieri *et al*., 2013; Maggisano *et al*., 2014). In rat Leydig testicular carcinoma cells (LC540) treatment of HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) in combination showed enhanced NIS expression both in transcript and protein level with subsequent improvement of radioiodine uptake (Maggisano *et al*., 2014). Evaluation was also done in liver cancer cells and correlated with p53 family member proteins showing increased NIS expression (Guerrieri *et al*., 2013).

In the context of BC, of the various known subtypes, more than 80% hormone receptor positive cases (estrogen and progesterone receptor) were reported to have natural expression of NIS protein, while around 65% triple negative breast cancer (TNBC) cases showed positive expression (Chatterjee *et al*., 2013; Tazebay *et al*., 2000; Wapnir *et al*., 2003). Taking these results forward, clinical studies have been carried out to verify the translational potential of NIS in malignant BC patients. Moon *et al.* (2001) reported significant $^{99m}TcO_4^-$ uptake in 4 out of 25 breast cancer patients by scintigraphic scanning method. Another important study by Wapnir *et al.* detected radioiodine uptake in metastatic BC by scintigraphic analysis using radioiodides ($^{123}I$ and $^{131}I$) or $^{99m}TcO_4$. However, surprisingly iodine uptake was noted in only 25% (2 out of 8) of NIS positive metastatic breast tissues (Renier *et al*., 2009; Wapnir *et al*., 2004). Therefore, current focus on achieving effective NIS gene therapy is to find potent modulators that could improve endogenous NIS expression and/ or function.

*Overcoming challenges of NIS gene therapy*

As mentioned above, exploiting endogenous NIS as a therapeutic target is limited by its ability to pump sufficient iodine inside cells, especially in non-thyroidal cancer tissues such as breast. This discordant is primarily due to lack of NIS expression on plasma membrane,

which is critical for its iodine transporter function delivering iodine inside the cell. In this regard, epidermal growth factor (EGF) was identified to localized NIS better on the plasma membrane in non-thyroidal cancers, while reverse localization was also obtained by treatment with a MEK-1 inhibitor, suggesting involvement of MEK-ERK signaling pathway in NIS localization (). Moreover, as far as improvement of NIS endogenous expression is concerned, trans-retinoic acid (tRA) was frequently studied as the major inductor in BC. Treatment with tRA showed significant increase in $^{131}$I mediated radioablation in MCF7 cell lines (Kogai *et al*., 2012). Besides, *in vivo* study using MCF7 xenograft mouse model showed enhanced radioiodine uptake sufficient for effective cell killing (Kogai *et al*., 2004). But due to short biological half-life, the effect of tRA exists in the system only for a limited time period and therefore requires frequent use during therapeutic intervention. However, frequent use of tRA caused cardio-respiratory distress syndrome in patients of acute promylocytic leukemia (Warrell, 1993). 13-cis RA can be used as a prodrug which finally gets converted into tRA inside the target cells. Experimental study has shown that although it induces NIS expression, the level of expression is much lower than tRA administration (Kogai *et al*., 2005). RAs operate by binding through two families of nuclear receptors, retinoic acid receptors (RARs), which are activated by both all-trans RA and 9-cis-RA, and retinoid X receptors (RXRs), which are activated by 9-cis-RA only. Upon binding of RAs, receptors get activated and bind to RA responsive elements in the promoter regions of target genes and work as ligand-dependent transcription factors (Alotaibi *et al*., 2010).

Primarily, NIS activation takes place by binding of RA to RARα/RXRβ receptor hetero-dimer followed by activation of the phosphoinositide 3-kinase (PI3K) pathway and the p38βMAPK pathway (Kogai *et al*., 2012). By administering PI3K inhibitor or by knock down of p85α (a regulatory subunit of PI3K) showed decreased RA induced NIS expression in MCF7 cells. Decrease in iodide uptake was also reported upon inhibition of AKT pathway in MCF7 cell lines (Kogai *et al*., 2012) suggesting involvement of PI3K/AKT pathway in NIS induction. Further, combination of tRA with several pharmacological compounds found to be

more effective in NIS gene induction in non-thyroidal cancers. Such combination therapies used drugs like hydrocortisone, dexamethasone (Dex) (Dohan *et al.*, 2006; Kogai *et al.*, 2005), troglitazone (a peroxisome proliferator–activated receptor γ, PPARγ, agonist) (Tanosaki *et al.*, 2003; Wei *et al.*, 2009), various histone deacetylase (HDAC) inhibitors (tricostatin A and sodium butyrate), and carbamazepine, an agonist of pregnane X receptor (Unterholzner *et al.*, 2006; Willhauck *et al.*, 2011). Some other compounds like prolactin, insulin, and insulin growth factor (IGF)-I and II were also used to stimulate NIS mRNA expression in BC cell line (Arturi *et al.*, 2005).

Moreover, the efficiency of radioiodine therapy in various cancers not only depends on NIS protein expression and its membrane localization, the factor that can't be ignored is biological half-life and retention potential of radioiodine in the tumor bed. In thyroid gland, due to iodine organification the biological half-life of iodine is sufficiently long, making radioiodine therapeutics effective for thyroid cancer treatment (Shimura *et al.*, 1997). However in lactating mammary gland approximately 20% of the iodine was trapped due to iodine oxidation by lactoperoxidase (LPO) expressed in alveolar cells followed by binding of iodine to various milk proteins (Etling *et al.*, 1984; Strum *et al.*, 1983). But this may be insufficient for radioiodine therapy in BC. So, studies are underway to improve iodine retention time in breast cancer cells for getting $^{131}I$ therapy effective. Combination treatment with all-trans RA and Dex has shown modest improvement in iodine retention in MCF7 cells. However, the exact mechanism is not known (Unterholzner *et al.*, 2006).

Furthermore, as NIS is highly expressed in normal thyroid tissues, while treating non-thyroidal cancers safeguarding thyrocytes can also be a major issue. However, this issue has already been addressed by selective down-regulation of NIS expression in thyrocytes by administering T3 and methimazole in combination (Wapnir *et al.*, 2004). Apart from thyroid and lactating mammary gland NIS is also expressed in several other normal tissues such as salivary gland, intestinal epithelium, lacrimal gland, stomach lining etc. that become barrier for NIS based imaging and treatment. Uptake in these normal tissues not only reduces therapeutic efficiency but

subsequently radioiodine damages of normal organ function as well.

**Clinical Experiences with NIS**

NIS based diagnosis and therapy is well known in thyroid clinics for several decades to identify and treat various thyroid diseases including cancer. In a recent case study after complete thyroidectomy in differentiated thyroid cancer patient, whole-body scans based on diagnostic or therapeutic doses of $^{131}$I can visualize various distant metastatic lesions. Nonetheless, extreme precautionary measure is required while analyzing the data because several false positive signals were also obtained (Ahn *et al.*, 2011). As per the NIH database (www.clinicaltrials.gov), at present there is no ongoing or completed clinical trials on NIS based therapy in non-thyroidal cancers. The reasons are indeed due to several such issues that are discussed above. So, investigators are actively investigating effective methods of modulation to enhance NIS expression and localization on plasma membrane to achieve the optimal efficacy. However, recently few clinical trials in prostate and ovarian cancer are attempted for NIS gene therapy. In a phase I clinical trial, prostate cancer patients received intra-prostatic injection of Ad5-yCD/mutTK(sr39)rep-hNIS followed by measuring hNIS expression over time through SPECT imaging after adenovirus injection. Positive hNIS expression was obtained in the patient's prostate gland suggesting non-invasive imaging of NIS gene is achievable and safe for humans (Barton *et al*., 2008; 2011). Moreover this study also proved the application of $^{131}$I in human for localized prostate cancer treatment. Recently a clinical study in ovarian cancer also showed promising observation upon intra-peritoneal administration of engineered measles virus to express NIS. NIS expression was confirmed in patient's tumor through $^{123}$I uptake using SPECT/CT scan (Galanis *et al.*, 2015). Further clinical evaluations are expected in the coming years to achieve successful NIS gene therapy application in non-thyroidal cancers.

**CONCLUSIONS**

Gene therapy in cancer has high potential because of recent advancement in genetic engineering in cellular and molecular level. In this arena, NIS gene gains importance in cancer for gene therapy because of its property to serve both

diagnostic imaging and therapeutics. Moreover to improve therapeutic effectiveness, NIS gene therapy is rapidly evolving in various non-thyroidal cancers particularly in BC because of lack of appropriate therapeutic options in hormone receptor negative patients. Although NIS gene therapy is already on track for various thyroid cancers, but for non-thyroidal cancers there are still various logistics that need to be addressed before successful clinical translation. Major concerns which need to be taken care are the expression level, protein localization on cell membrane and lower retention time of radioiodine in cancer tissues. So, optimal strategies are yet to be developed to improve radioiodine uptake and retention by modulating NIS gene expression in non-thyroidal cancers. Expectedly, the progression in recent basic research unraveling NIS biology in the field of gene therapy would develop right strategies of treatment to care devastating cancer.

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## CONFLICT OF INTEREST

The authors claim no conflict of interest.

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Review

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# Microbiota in Immune Pathogenesis and the Prospects for Pre and Probiotic Dietetics in Psoriasis

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Psoriasis is a common autoimmune inflammatory disease wherein pathogenesis is advanced by fundamental genetic predisposition/s in concert with environmental triggers. Inflammation in psoriasis may represent efforts of innate immune system to target pathogens for restoring immune homeostasis. Aberrant microbiota may resist elimination efforts by shear advantage of several fold gene pool as compared to the host. The microbes deregulate gene expression by the molecular insults targeting host immune system. Role of microbiota in autoimmunity dictates establishment of microbiome homeostasis and suppress host immune response; as a treatment approach. Dietary prebiotics and probiotics are of particular interest for prevention and amelioration of autoimmune inflammatory diseases, due to their potential to foster healthy host-microbiome relationship. The rational dietetics aims towards balancing friendly versus enemical microbes via manipulation of gut environment and modulation of immune system to improve regulation of inflammatory and autoimmune mechanisms.

## INTRODUCTION

Increasing prevalence of autoimmune disorders and global epidemic of 'modern age' chronic inflammatory diseases due to changing lifestyle and environment is currently witnessed (Pandey *et al*., 2014). A genetic predisposition underlies autoimmune diseases, with immune, hormonal and environmental factors contributing to the clinical manifestations. Clinical manifestations are restricted to susceptible individuals, although the autoimmune processes have wider occurrence. Genetic predisposition influences the disease precipitation by exposure to environmental triggers as sunlight, diet, allergens, infectious agents and several other environmental insults. Infectious agents commonly trigger autoimmune diseases. Microbial products can aggravate T cell responses to self as well as non self antigens. Environmental factors may shift the balance of T cells

**Key words:** Psoriasis, Autoimmunity, Microbiota, Dysbiosis, Endotoxins, Epigenetic, Superantigens.

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between inflammatory interferon gamma producing Th1 cells and IL-4 and IL-5 producing Th2 cells in an individual. Lifestyle factors apparently affect immune function. Interaction between dietary factors and other exposure/s are current significant research areas of pathogenesis and therapeutics of autoimmune disorders (Pandey and Pandey, 2013).

The microbial flora in the gut interacts with diet and influences the host immune cells with mutual benefit to both the microbes and the host (Lee and Mazamanian, 2010; Lee *et al*., 2010). This aspect has major implication for prevention and mitigation of psoriasis, the most common immune mediated inflammatory disease.

**The Gut Microbiome**

The gut mucosal immune system is the largest lymphoid organ in our body, intricately involved in regulation of immunity and inflammation characterizing autoimmune disorders. Gut immune system is strongly subject to changes by dysbiosis or imbalance among microbial species (Maynard *et al*., 2012). Change in population of singular species of segmented filamentous bacteria in the gut may markedly influence emergence and stability of specific T lymphocyte subsets (Prakash *et al*., 2011). Biochemical interactions of colonic microbes and immune cells have implications for immune homeostasis. Examples include, action of serum amyloid A on dendritic cells (Ivanov *et al*., 2009), ATP mediated activation of dendritic cells (Atarashi *et al*., 2008), induction of TGF-beta expression by gut epithelial cells (Atarashi *et al*., 2011), bacillus fragile derived polysaccharide A action on dendritic cells (Mazamanian *et al*., 2005) and TR-2 regulatory T cell function (Round *et al*., 2011). Changes in gut microbiota in response to diet, sanitation, antibiotic use, environmental chemicals, etc. can influence and impact maturation and balance of immune responses.

**Dysbiosis and Immune Disorder**

Innate immune system provocation in autoimmunity involves a crucial role of the Toll like receptors. These recognize specific forms of microbial nucleic acids and bind them to induce proinflammatory signals. The TLRs also recognize certain self antigens and mediate autoimmunity against cells with parallel expression of proinflammatory cytokines (Trivedi and Greidenger, 2009). The common proinflammatory autoimmune phenotypes are due to CD4+ T helper lymphocytes. Organ specific autoimmunity is driven by cell mediated immune responses aimed to attack intracellular foreign element. The Th1 cytokines IL-2 and interferon-gamma predominance foster development of such

responses, as in psoriasis (Smith and Germolec, 1999; Street and Mossman, 1991). The Th17 lymphocyte phenotype contributes to autoimmunity through procuring neutrophil chemo-attractant and activator cytokine IL-23 (Wild *et al*., 1994). Several intracellular signal transduction pathways in the Treg (Suppressor) lymphocytes may be deregulated leading to inappropriate activation of naïve T cells and persistence of autoreactive cells in organ (Jain *et al*., 2010). Altered quantities of STAT-3 (signal transducers and activators of transcription) influence autoimmunity in varied ways. There is direct selection, favouring Th-17 pro-inflammatory phenotype over the immune-inhibitory Treg phenotype of lymphocyte subsets. STAT-3 also influences vital T-cell biology of growth and survival as well as transcription of pro-inflammatory genes (Egwuagh, 2009).

The microbes may initiate autoreactivity by several mechanisms (Ercolini and Miller, 2009). Infection induced general proinflammatory environment serves as a bystander to promote deregulation of immune response and modification of endogenous proteins to autoantigens. Alternatively some microbial antigens may mimic structurally homologous self peptide of the host which initiates immune response. The increasingly emphasized mechanism however, is production of super-antigens by certain microbes. These nonspecifically crosslink MHC-II to T cells primed to other antigens including self-antigens, leading to unintended stimulation and massive release of cytokines (Freidman *et al*., 1991; Schiffenbouer *et al*., 1998).

Intestinal dysbiosis generates endotoxin-peptidoglycan super antigens inducing autoimmune/inflammatory pathology in psoriasis. Immune response is directed at toxins produced by microorganisms in the gut, and psoriatic patients exhibit positive skin test to gut bacterial antigens (Baker *et al*., 2006a; 2006b; Gyurcsovics and Bertok, 2003; Karotkii and Peslyak, 2005; Qayoom and Ahmed 2003; Stenina *et al*., 2003). Gut and skin colonization by *Staphylococcus aureus*, malassezia, and candida, etc. cause exacerbation in psoriasis (Fry and Baker 2007). Autoimmune reactions can be advanced or blocked by commensal bacteria affecting the innate and adaptive arms of immune responses and interlinking mechanisms. Whether immunity and autoimmunity is affected by specific or multiple lineages of microbes that may shift the homeostatic balance toward reduced or exaggerated reactivity in host-microbiota interaction, is yet unsettled (Chervonsky, 2013).

New genomic understanding indicates collective metagenome (Interactive

microbe-host genomes) as determinant of outcome of host-microbiome interactions. Diverse microbial metabolites may affect expression of genes associated with immune responses and autoimmunity. Microbiota accumulates incurring adaptations to persist, with the genes impacting the disease process. Success in reversing autoimmunity by reduction of microbes that have evolved capability to block vitamin-D receptors and thus evade immune-elimination supports the view (Proal *et al*., 2009). Huge load of metabolites resulting from over million microbial genes, are juxtaposed to interact with small number of proteins made by human genes. Immune function thus is manipulated (Honda and Littman, 2012) Genetic predisposition leads to adverse consequences following interaction with other entities. Altered cytokine profile can change cellular milieu and xenobiotic exposures may vitiate the micro-world of immune cells. Altered gene expression as a result of an epigenetic change and surge of proinflammatory mediators weakens immune regulation and tolerance. The risk of autoantigen availability and consequent autoimmune disease therefore increases (Pillai, 2013).

**Involvement of Gut-brain Neuro-immune axis**

Dendritic cells in the gastrointestinal (GI) tract send processes throughout the gut epithelium in the lumen to interact with microbes. The signals to humoral immune system to produce immunoglobulin A secretion prevails (Corthesy and Spertini, 1999). The secreted IgA checks microbes from penetrating gut epithelium. The dendritic cells are in close proximity to the nerves in GI tract and their function is modulated by sensory neuropeptide CGRP (Calcitonin gene related peptide) (Hosoi *et al*., 1993). The brain is informed about microbiota via the vagus nerve (Gochler *et al*., 1999). Bacterial endotoxins or inflammatory cytokines like IL-1β and TNF-α may stimulate the vagus nerve. The vagal reflex in response, suppresses proinflammatory cytokine release by intestinal macrophages (Borovikova *et al*., 2000).

The gut-brain axis modulates the feeding behavior as well (Bercik *et al*., 2009). Peripheral afferent nerves transmit "danger" signals and elicit neural reflexes, regulating immune responses. Prototype inflammatory reflex operates through afferent sensory and efferent motor vagus nerve fibers (Tracey, 2009). The central projections regulate hypothalmic-pituitary-adrenal (HPA) neuro-humoral axis and causes glucocorticosteroid release. The afferent vagal activity triggered by endotoxins and cytokines, also sends efferent signals to thymes and the splenic nerve (Rossa-Ballima *et al*., 2008). Spleen is the primary target for

signals in the efferent pathway of vagal antiinflammatory reflex. Over 90% of systemically released TNF-α during early endotoxinaemia, is of splenic origin. Vagal stimulation attenuates TNF-α release. Vagus effect is mediated through adrenergic splenic nerve activation and β-2 adrenocepters mediate inhibition of TNF-α release (Vida *et al*., 2011). The mechanism of precipitation and aggravation of psoriasis lesions by β-adrenergic blocking drugs is thus explained. The innervating vagus nerve fibers coordinate with the gut microbiome via bidirectional communications (Lee and Mazmanian, 2010).

**Dietetic Management of Gut Microbiota**

Diverse commensal bacteria reside in the gut. Individual species appear to have distinct and opposite roles in gut immune response. Certain commensal microbes preferentially drive regulatory Treg lymphocyte development, while others promote pro-inflammatory Th1γ cell development in gut lymphoid tissue (Kamadaand Nunez 2013). Altered microbiota associates with several inflammatory diseases (Kamada *et al*., 2013).

Microbiota in gut serves a number of nutritional health effects. The composition and performance is influenced by diet, as a key factor. Dietetic strategy attempts to suppress harmful bacterial species while stimulating beneficial bacteria. Such a strategy implies selective consumption of probiotics and/or prebiotics and diet rich in fiber content.

**Prebiotics**

Most plant origin foods contain dietary fiber. The fibers undergoing bacterial degradation include polysaccharides e.g., resistant starch, pectin, innulin, guar gum and oligosaccharides. Structural polysaccharide like cellulose and lignin are insoluble and are not degraded by bacteria e.g., wheat bran. Such components have ability to hold water and thus increase mass of stool. This facilitates motility and cleansing of gut microbial mass. Soluble fibers also increase fecal output and promote bacterial biomass via fermentation. Prebiotics are no digestible food components that selectively enhance growth and/or activity of one or limited bacterial species with beneficial health consequences. Prebiotic has to remain undigested and unabsorbed in upper segment of GI tract. The majority of prebiotics are oligosaccharides, however some polysaccharides also serve as substrates to colonic bacteria and stimulate their activity. Prominent prebiotic activity is seen with non-digestible oligosaccharides including xylo-oligosaccharides, galacto-oligosaccharides and isomalt-

oligosaccharides (Van Loo *et al*., 1999). Anaerobes constitute over 99% of fecal flora. These break down the available carbohydrate substrate to short chain fatty acids, acetate, propionate, butyrate and gas hydrogen and carbon dioxide. Propionates and acetates are absorbed and contribute to the fuel resource of the body. Butyrate is a preferred energy resource for colonic epithelium and plays a role in proliferation and differentiation (Litvak *et al*., 1998). The hydrogen generated through fermentation reactions is primarily used by methanogenic, acetogenic or sulfate reducing microorganisms (Gibson *et al*., 1990).

Stimulation of Bifidobacteria and Lactobacilli is advantageous due to their immuno-modulatory abilities and inhibitory potential against pathogens. These reduce ammonia formation and lower blood cholesterol, and serve to restore gut microbiota damaged by antibiotics (Goldin, 1998). Selective stimulation of indigenous beneficent microbe strains, impart antimicrobial potential of prebiotics. The beneficent microbes selectively possess exo-glycosidase enzymes that enable utilization of oligosaccharides (Perrin *et al*., 2001). The uptake and intracellular metabolism by the microbes as an alternate strategy (even by non-beneficent microbe) is a possibility. Prebiotic selection needs refinement for avoiding the later alternate pathways. Antimicrobial potential is particularly vested in smaller prebiotic molecules e.g., chito-oligosaccharides (Vishnukumar *et al*., 2005). Bacterial species have different preferences for energy substrates. Diet is a strong direct means of influencing gut microbial colonization. Dietary fiber effectively causes major shifts in composition of gut microbiota and directly affects mucosal immune system. Fiber therefore improves chronic inflammatory disorders and systemic immune responses. Anti-inflammatory potential is contributed through short chain fatty acids generated upon microbial fermentation of prebiotic components (Huda-Faujan *et al*., 2010). Butyrate is richly produced from resistance starch, soluble fiber, and innulin foods and increases the regulatory Treg lymphocyte percentage with reduced production of interferon-γ. As a consequence, there is down regulation of inflammation (Vieira *et al*., 2013). Higher levels of butyrate causes activation of nuclear transcription factor and peroxisome proliferator activator receptor gamma PPAR-γ (Luhrs *et al*., 2002; Schwab *et al*., 2006). PPAR-γ activity inhibits proinflammatory pathways like STAT, AP-1 and $NF_KB$ pathways, specifically desired in psoriasis management (Sertzing *et al*, 2008).

Acetate is produced in greater abundance than butyrate following

fermentation. Acetate levels are raised more in circulation than in the gut due to absorption. Immune cells bear specific G-protein coupled receptors for binding the small chain fatty acid ligands. Specific GPR43 receptor mediated protection against colitis through induction of Fox P3+ IL-10 producing regulatory Treg cells has been demonstrated (Smith *et al*., 2013). Prebiotics inhibit pathogen adherence to gut epithelium, with positive effects on lipid metabolism and stimulation of mineral (especially calcium) absorption in colon, through influencing the gut microbiota (Gibson and Roberfroid, 1995).

**Polyphenolic Bioactive Food Constituents**

Colonic microbiota serves as primary agents for metabolism of polyphenolic dietary constituents. These are esters, glycosides and polymers contained in fruits and vegetables and bear protective antioxidant potential. Citrus fruits, apples, grapes, berries, wine, tea, soy and many vegetables including onion are rich sources of dietary polyphenols comprising complex mixtures. The nature of the gut microbiome therefore, determines extraction of their bioactive antioxidant and anti-inflammatory principles. Polyphenolics like isoflavones and flavonones are absorbed to a small extent. Proanthocyanidines and anthocyanidines are obligatorily metabolized by gut microbiota (Manach *et al*., 2005). Strategic modulation of composition of gut microbiota may enhance utilization and bioavailability of polyphenols and their potential health benefits. Synergistic benefit of simultaneous oligosaccharide consumption is observed with isoflavones (Mathey *et al*., 2004; Piazza *et al*., 2007).

**Probiotics**

Two bacterial phyla, Bacterioides (bifidobacteria) and fermicutes (lactobacilli) comprise over 90% of the gut microbiota (Mariat *et al*., 2009). These produce large number of vitamins including the B group vitamins; synthesize amino acids; and carry out bio-transformation of bile; and ferment undigested fiber and mucus. Beneficent microbes produce antimicrobial substances and promote mucin secretion and directly interfere with pathogen adherence to the epithelium (Rogier *et al*., 2014). The bifidobacteria and lactobacilli can be introduced in the gut and encouraged to multiply as probiotics by supplementing prebiotic rich diet. Administration of these probiotic strains in healthy individual enhances mucin and nonspecific IgA secretion and the phagocytotic potential of surveillance cells.

Thus, probiotic strengthens the gut barrier and opposes entry of foreign

antigens. Attenuation of proinflammatory responses adds to this. Probiotics compete for nutrients at the site of attachment to gut epithelium and inhibits colonization by pathogens with simultaneous release of antimicrobial products. Generation of lipopolysaccharides and peptidoglycans detrimental to the host is checked by probiotic mechanisms (Tlaskova-Hogenova *et al*., 2004). A major role for peptidoglycans is emphasized in psoriasis pathogenesis (Baker *et al*., 2006). The development of regulatory T cells, the Type1 and Type2 helper T cell and Th1γ helper cells are all subject to signals by intestinal microbiota. The pro-inflammatory responses attenuated by probiotics include IL-8, MCP-1, MIP-1 and RANTES, proinflammatory cytokines and lipid mediators evoked by pathogens. RANTES activation is of major pathogenic significance in psoriasis (Raychaudhuri *et al*., 1999).

Stable health promoting relationships between host gut and microbiota is crucially determined by pattern recognition receptors viz. Toll like receptors (TLRs) and Nod like receptor (NLRs) (Abreu, 2010). Microbe associated molecular patterns signal to affect epithelial cytoprotection, survival/ proliferation pathways and barrier function (Rakoff-Nohoum *et al*., 2004). TLR activation upregulates proinflammatory mediators facilitating immune defense. The NLRs are present in the cytoplasm of immune cells and their stimulation by commensal associated signals regulate inflammatory responses, contributing to gut homeostasis (Yeretssian 2012). Disturbed interaction of microbiota with pattern recognition receptors underlies diseases with exaggerated inflammation (Lavelle *et al*., 2010; Maynard *et al*., 2012). An investigation of mechanism by which specific probiotic strain triggers reaction can help to indicate appropriate choice of probiotic for prophylactic use in diverse inflammatory diseases. The probiotic benefit is external to gut through complex microbe-immune system interaction in immune mediated inflammatory diseases. The gut-brain axis and inflammation reflexes have a bearing in this context.

Dysbiosis of gut microbiome may be a secondary consequence of a primary adversary that must be diagnosed and managed. The metabolic phenotypes of individuals determine the composition of gut microflora independently of dietary pattern or even genotype (Serino *et al*., 2012). Long term consumption of high fat diet impacts the microbiota directly, and indirectly through alteration of redox state. Antioxidant dietary supplements improve gut microbiota profile (Espley *et al*., 2014). A protective potential of gut microbiota against pathogen invasion is promoted by prebiotic combination with

other bioactive plant principles and quality proteins in the diet. Lupine fermentation is noteworthy in this regard (Berthkiene *et al*., 2013). Probiotic administration by rectal route is far superior to oral route for successful immuno-modulation (Matthes et al, 2010).

**The Pathogenic Gut-skin Linkage in Psoriasis**

Cytokinaemia and exaggerated inflammation in psoriasis is crucially linked to absorption of endotoxins from pathogenic gut bacteria (Gyurcsovics and Bertok, 2003). A strong evidence of defective barrier function of gut is observed (Scarpa *et al*., 2000). Immuno-pathologic process in psoriasis extend from gut to skin (Michaelsson *et al*., 1997). The evidence is compelling to address issues of gut barrier integrity and dysbiosis as a rational consideration in psoriasis therapy. Psoriasis therapies are conventionally focused on managing the consequences of immune mediated inflammatory pathology and causes several adverse effects. Regular incorporation of prebiotic and probiotic dietetics is a rational consideration, neither too expensive nor unsafe. Its significance particularly appeals for prophylaxis in individuals with familial predisposition, and subjects bearing other heightened risk factors (Gupta *et al*., 2013). Evidence based personalized pre- and pro-biotic dietetics in management of psoriasis has appeal. This is challenging however, microbiome management is subject to individual contexts and not amenable to ordinary laboratory means. Metagenomic investigations may be a solution to match the indications, with appropriate dietetic address. This may comprise a rational and lead to better quality management for psoriasis and autoimmune inflammatory diseases at large.

## CONFLICT OF INTEREST

The authors claim no conflict of interest.

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