Head and Neck Squamous Cell Cancer: Biology (II) and Translational Methods

Anupam Mishra · Amita Pandey · Xiaolin Nong

Abstract

The essential steps implicated in carcinogenesis are acquisition of autonomous proliferative signaling; inhibition of growth inhibitory signals; evasion of apoptosis; immortalization; angiogenesis; tissue invasion and metastasis. A considerable progress in understanding the process of carcinogenesis has further stimulated the high throughput translational methods and systems biology approach to revolutionize this field of cancer biology. The era of molecular targeted therapy has dawned and would soon replace the more 'toxic' classical 'broad-spectrum' cancer chemotherapy. This review summarises the steps of carcinogenesis and the concepts involved in translational methods, systems-biology and molecular targeted therapy.

Keywords Molecular targeted therapy · Carcinogenesis · Systems-biology

Introduction

The cancer is an uncontrolled proliferation of cells by-passing the inhibitory signals. This can result either from acquisition of autonomous proliferative signaling or inhibition of normal inhibitory signals leading to evasion of apoptosis (pre-programmed cell death) and immortalization. The progression of cancer then requires acquisition of nutrient blood supply to ultimately invade the surrounding tissues and cause distant metastasis. Following are the six important steps for carcinogenesis as described by Hahn et al [1], and Hanahan & Weinberg [2].

Step I: Acquisition of autonomous proliferative signaling

A normal cell requires continuous cell signaling to maintain homeostasis. Such growth signals are received at the cell surface by membranous receptors that through their intracellular domains stimulate a variety of signaling cascades and subsequently the signal is ultimately transmitted to the nucleus. The exogenous signals may be in the form of small molecules (growth factors), cytokines, cell-cell interaction, extracellular matrix components etc. The independence of a cell from exogenous growth signaling marks one of the hallmarks of malignancy. The autonomy may also involve the intracellular signaling pathways as such.

The epidermal growth factor (EGF) axis has been implicated in the HNSCC development. This receptor signals multiple oncogenic pathways including RAS, JAK-STAT, MAPK, PI3K-AKT. Most studies concerned with the role of EGF, EGF-receptor (EGFR) and transforming growth factor (TGF) in HNSCC development have focused on the quantification of expression of DNA, RNA, or proteins along with their clinical correlation. Although inconsistent the results still indicate EGF axis to be up-regulated in the majority of HNSCC. For example, Sauter et al [3] in an immunohistochemical analysis of SCC of tongue base found that EGFR & TGF were normally expressed in normal squamous mucosa adjacent to the tumour, but the tumour

A. Mishra¹ MS, DNB (✉) · A. Pandey² MS, DM · X. Nong³ MD, PhD

¹Currently a Visiting Faculty and International
UICC (American Cancer society)

Fellow at the Department of Head and Neck Surgery,
UT MD Anderson Cancer center,
Houston, USA.

²Assistant Professor (Gynaecologist and Genetic Specialist),
Department of Obstetrics and Gynaecology,
K.G. Medical University,
Lucknow.

³Associate Professor in Oral and Maxillofacial Surgery,
Guangxi Medical University, China, and currently a
Post-doctoral fellow in department of Head and Neck Surgery,
MD Anderson Cancer Center,
Houston, USA.

A-1/ 19, sector H,
Aliganj,
Lucknow (UP)
India.
E mail: amishra_ent@yahoo.com

itself showed increased (60% & 35%) expression. However this over-expression did not show any impact on the survival. Similar observation was seen with the study of Furata et al [4] with the tumours of nose and paranasal sinuses. On the contrary Maurizi et al [5] showed over-expression of EGFR in poorly differentiated laryngeal cancers when compared to the normal mucosa. But in this study however, the 2 year survival of patients with negative EGFR expression was more (82%) as compared to those with positive expression (58%). Grandis & Tweardy [6] investigated the role of TGF and EGFR in the pathogenesis of HNSCC by looking on the m-RNA levels in the tumour tissue and the normal mucosa from the patient as well as from the normal controls. They found comparable levels of m-RNA of both the ligands and receptors in tumours and normal mucosa of the patient suggest that environmental influences such as tobacco and alcohol usage, lead to up regulation of growth factor production and receptor expression that may play a role in tumour development. Grandis & Melhem [7] quantified EGFR and TGF via immunohistochemical staining and increased levels of both were found to be independent predictors of disease free survival. Other studies [8,9] also support this observation.

Maxwell et al [10] examined EGFR expression in 2 HNSCC cell lines by immunoprecipitation and found that the rapidly proliferating cell line showed five fold higher levels of EGFR and greater intrinsic tyrosine kinase activity. The tyrosine kinase enzymatic activity is a part-and-parcel of EGFR signal transduction and was found to increase in immunoprecipitates from both the cell lines by the addition of exogenous EGF.

EGFR and its ligands have been implicated in invasion and metastasis as well and Pornchai et al [11] have shown an association between its up-regulation and activation of matrix metalloproteinase (MMP) in HNSCC cell lines. Shibata et al [12] in addition also showed an up regulation of urokinase type plasminogen activator.

Step II: Inhibition of growth inhibitory signals

The orchestration of normal cell division, growth and differentiation, takes place amidst a proper balance between the proliferative and inhibitory signals. Once this tender balance is altered either by enhanced proliferative signaling or decreased inhibitory control, the cell enters into uncontrolled growth phase. The cell cycle consisting of 4 phases (G1, S, G2, M) are tightly controlled by cyclin and their regulators – cyclin dependent kinases (CDKs) and cyclin kinase inhibitors (CKIs). Factors affecting cyclin, CDKs, CKIs will have a significant effect on cell growth and are potential carcinogenic mediators.

The well known tumour suppressor gene p53 resides on the short arm of chromosome 17 and acts through regulation of gene transcription. It protects the cell from DNA damage from radiation or chemicals by arresting the cell cycle so

as to facilitate either DNA repair or pre-programmed cell death [13,14,15]. More than 92% of mutations affecting p53 occur at the functional sites of the gene impairing the cells ability to repair its DNA which leads to genomic instability of the cancer cells with additional alterations in oncogene and tumour suppressor gene products. This may further prevent apoptosis in response to DNA damage that may give rise to radiation- as well as chemo-resistance. Somers et al [16] observed G to T transversions in p53 over-expression among 10 of 13 tissue specimen and 13 of 13 cell lines. Since such transversions are known to result from interaction of DNA with benzopyrene (a component of cigarette smoke), this study suggests a possible mechanism through which smoking may contribute to carcinogenesis. Shin et al [17] from MD Anderson Cancer Center have shown a steady increase in p53 abnormalities in the progression of heads and neck tumours. Such abnormalities have been seen in 19% of normal epithelium, 29% of hyperplastic lesions, 45% of dysplastic lesions and 58% of invasive cancers demonstrating p53 over-expression. It was further shown that a significant association between p53 expression and genomic instability exists suggesting that p53 alterations may lead to the accumulation of genetic events during carcinogenesis. There is an accumulating evidence of the role of human papilloma virus as a causative agent in a subset of HNSCC and the mode of action is through HPV E6 & E7 oncoproteins that bind and thereby deactivate p53 functioning [18]. Similarly another cellular protein mdm-2 binds and thereby inactivates transcriptional activation domain of p53 [19]. Haupt et al [20] have further shown this mdm-2 to have a role in degradation of p53 as well. Ganly et al [21] have shown that 95% of p53 inactivation occurs either by mutation, HPV infection or mdm-2 over-expression.

The INK4 family of tumour suppressor genes are mainly known to act on G1 phase of cell cycle. These include INK4B (p15), INK4A (p16), INK4C (p18) and INK4D (p19). Serrano et al [22] demonstrated that p16 binds to and inhibits the enzyme cyclin-dependent-kinase 4 (CDK4), which is important in enabling cells to pass through the cell cycle. Hence a fine orchestration between cell cycle inhibitor and cell cycle activator is disrupted by gene expression to result in carcinogenesis. Reed et al [23] showed the absence of p16 activity in 83% of primary tumours through genetic or epigenetic mechanisms. Alterations of p16 gene coding region have been found in various pre-malignant and malignant lesions of the oral cavity [24,25,26]. The frequent observation of p16 gene mutations in dysplastic as well as neoplastic oral lesions suggests it to be an early event in carcinogenesis.

Cyclin D1 amplification has been identified in up to 64% of HNSCC [27]. Over-expression of cyclinD1 in 68% of tongue cancers statistically correlated with reduced disease free survival and over all survival [28]. Mineta et al [29] on the other hand correlated high cyclin D1 expression with lymphatic metastasis. Likewise the role of cyclin D1 has

been documented at various subsite in the head and neck. Michalides et al [30] have shown that over-expression of cyclin D1 protein in HNSCC is an independent prognostic factor for recurrence. Nakashima & Clayman [31] showed that transfection of antisense cyclin D1 into head and neck cancer cell lines caused decreased in vitro growth rates and decreased tumorigenicity in a nude mice model. This suggests the possibility of new gene therapy approach.

Step III: Evasion of apoptosis

Much discussion has been done in the earlier section of the article regarding apoptotic cascades and its significance. The evasion of the pre-programmed cell death seems to be an essential hallmark in carcinogenesis. The cellular effectors or caspases can be divided in three groups: (1) Non-apoptotic caspases [1, 4, 5, 11, 12, and 13] having no role in apoptosis. (2) Initiator caspases [8, 9, and 10] function as initiators and amplifiers of apoptosis. (3) Effector caspases [2, 3, 6, and 7] are the ultimate effectors of apoptosis. Upon activation the initiator caspases further activate effector caspases that then cleave cellular proteins, cytoplasmic organelles, chromosomes etc leading to the morphological changes typical of apoptosis. Various chemotherapeutic agents are known to induce apoptosis by up-regulating caspases. For example 5-fluorouracil up-regulates caspases [1, 3 and 8] while cisplatin selectively upregulates caspases [3, 8 and 9].

As mentioned previously the molecules with a regulatory role in apoptotic pathway belong to Bcl-2 family and are responsible for regulating the release of cytochrome C from mitochondria. The best studied in HNSCC are Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) but the findings involving these have varied. For example Bcl-2 over-expression has variably been associated with better [32] or worse [33] overall survival.

The transcription factor NF-kappa B has also been shown to suppress apoptosis through transcriptional modulation of the expression of multiple apoptosis inhibitory proteins such as c-FLIP, TRAF-1, TRAF-2, c-IAP1, c-IAP2, Bcl-xL, IEX-1 and XIAP [34]. It has been shown that the activation of NF-kappa B decreases p53 dependent apoptosis [35] as well as TNF- α mediated apoptosis [36]. This further emphasizes the therapeutic potential of inhibitors of NF-kappa B signaling.

Step IV: Immortalization

Normal cells can undergo 60-70 mitotic cycles thereafter the ensuing chromosomal disarray results in severe crisis and ultimately cell death. Cancer cells however acquire an infinite ability to multiply through a process termed immortalization. The immortalization of cancer cells are often related with the roles of telomeres. Telomeres or the chromosomal 'caps' contain six base pair sequence TTAGGG

repeated many thousands of times. These serve at least three critical function: (1) To protect chromosome ends from enzymatic degradation and abnormal fusion reactions; (2) To serve as a buffer zone to protect against the 'end-replication' problem; (3) to serve as a scale for mitotic age (the divisional clock). Telomeric repeats are lost with each round of cell division, leading to a decrease in mean telomerase length. After a certain length, the short telomeric DNA sequences trigger entry into senescence pathway and ultimately leads to apoptosis. Only those rare cells which engage mechanisms that stabilize telomere length are capable of indefinite proliferation (or resulting in cancer). In 90% of human cancers, telomere stabilization is achieved by reactivation or upregulation of telomerase. Telomerase is an RNA-protein complex that utilizes its RNA as a template for addition of TTAGGG repeats, thereby compensates losses due to end-replication problem. Hence for a cancer cell the telomerase activity has been reported to be much higher (at least 6 fold compared to their benign counterparts) that accounts for its immortality. Recent studies have focused the telomerase as a diagnostic, therapeutic and prognostic target.

Step V: Angiogenesis

Normally there occurs a balance between pro-angiogenic (VEGF, FGF, PDGF) and anti-angiogenic (interferons, endostatin, angiostatin, thrombospondins) factors. Tumour progression is characterized by an imbalance to enhance neovascularization. Tumour can attain a maximum size of 1 cubic millimeter if it has to depend on simple diffusion for its nutrient requirements and hence for it to attain a substantial size, the acquisition of nutrient blood supply through angiogenesis is essential. This neovascularization appears to be regulated by both stimulatory as well as inhibitory factors and is considered a multi step process that include degradation of extracellular matrix (ECM), endothelial cell proliferation, migration and assembly of endothelial cells into higher order structures. Studies quantifying angiogenesis in terms of the microvessel density using endothelial cell markers (factor VIII, CD31, CD34) have yielded conflicting results in HNSCC. Hence such a consensus has not yet been established. However the studies indicate that angiogenesis is important for HNSCC biology and may prove to be a clinically important prognostic tool as well as a target for biologic therapy in future. The most studied positive regulator of angiogenesis is vascular-endothelial-cell-growth-factor (VEGF) that has a pleiotropic angiogenic effects. It is a potent endothelial cell mitogen, promotes cell survival & motility, endothelial cell organization and permeability across endothelial cell layers. O-charoenrat et al [37] have demonstrated an association between expression of VEGF family members VEGF-A & VEGF-C in SCCHN with increased risk of lymph node metastasis. So far the studies incorporating VEGF in HNSCC have yielded conflicting results and have shown no prognostic significance

in terms of the tumor stage, invasive behavior and survival. Hence there is no consensus of the role of VEGF in pathogenesis of HNSCC. Apart from VEGF, the angiogenesis initiating signals are exemplified by interleukin-8 [38] and fibroblast growth factor (FGF 1/2) [39]. The inhibitors of angiogenesis signals are thrombospondin 1, which binds to CD36, a transmembrane receptor on endothelial cells. Angiogenesis factors associated with SCCHN development has been used as therapeutic targets and currently only used experimentally.

Step VI: Tissue invasion and metastases

The three steps seem to be critical for tissue invasion: (1) alteration of tumour cell interaction to the basement membrane, (2) degradation of the extracellular matrix, and (3) tumour cell migration. Although the loss of basement membrane has not been correlated with invasive behaviour of HNSCC [40,41], but a wide variety of proteolytic enzymes and there specific inhibitors are reported in the development of tissue invasion and regional metastasis of HNSCC.

Cadherins are the proteins connecting the adjacent epithelial cells. The classical example is E-cadherin which is responsible for epithelial cells adhesion as well as polarity. Since loss of E-cadherin is documented in carcinogenesis, it is considered as tumor suppressor gene. Hence the inhibition of the normal growth suppression and antiproliferative activity results in uncontrolled proliferation.

Invasion of basement membrane is critical for local tumor spread. Matrix degradation metalloproteinases (MMPs) are instrumental in promoting the degradation of basement membrane. The most studied member of MMPs is MMP-9 that is shown to be up-regulated with tumor progression in terms of local spread and distant metastases.

Due to the loss of contact inhibition, polarity along with alteration in cytoskeleton, the tumor cells migrate through

intercellular spaces of basement membrane into the extracellular spaces. ‘Twist’ gene has been implicated in this process.

Cancer translational research in post genomic era

The basic research in cancer biology relates to its components (structure and function), organization (genetic networks and protein pathways) and diversity (heritable and evolutionary variation). On the other hand, translational research in biology-applied-to-health relates to genetic contribution to cancer, genetic contribution to health, genetic predictors of risk factors and chemical genomics or therapeutics. The conclusion of the human genome project has highlighted the molecular diversity for carcinogenesis in terms of multigene networks, multifactorial processes leading to complex diseases. The high throughput analytical systems involving robotics can delineate the genetic pathways from DNA level (DNA sequencing, SNP analysis), through RNA transcripts (DNA-microarray, RT-Q-PCR), to proteomics (mass spectrometry – MALDI). Microarrays consist of ordered array of hundreds to thousands of DNA molecules of known sequence representing individual genes and permits the simultaneous detection and measurement of thousands of gene in a small sample of DNA or RNA. The uses of microarray and RT-Q-PCR are shown in Table 1. Hence to plan a marker gene study, three steps are essential. Firstly a gene screening through differential display or DNA microarrays (chips) either qualitative or semi-quantitative. Secondly a cluster analysis to identify the predictors and surrogate end point markers. Thirdly a quantitative analysis of gene expression by high throughput ‘real-time’ Q-PCR validation and analysis. Although DNA microarray has identified genomic signatures of lymph node metastases and enhanced detection of occult metastases, further evaluation of these current technologies is in progress.

Table 1 Summary of Microarray and RT-Q-PCR

	Microarray - based Analysis	Q-PCR Based Analysis
Advantages	<ul style="list-style-type: none"> Exceptionally broad - based profiling capacity (up to 40,000 genes per chip). Access to global changes in gene expression and pathways Easy to apply (commercial and custom made platforms) Most useful for “gene hunting” type of Biomarkers 	<ul style="list-style-type: none"> Extremely sensitive and specific Very accurate quantitation of gene expression Applicable to very small samples (tolerant to RNA degradation) High throughput capacity (100’s to 1000s) Easy applicability - assays, instrumentation and analysis Most useful for Biomarker Analysis
Limitations	<ul style="list-style-type: none"> Need for independent validation (specificity) Semi-quantitative Limited throughput 	<ul style="list-style-type: none"> Applicable to the analysis of “known” genes Not much useful for “gene hunting” Fewer genes can be characterized

Systems biology approach for cancer

This novel approach is a combination of computational and experimental systems biology approach that can facilitate our understanding of complex biologic mechanisms of cancer, by systematically integrating the existing enormous data obtained through high-throughput genomics, proteomics, and metabolomic technologies. The computational systems through mechanistic dynamical simulations of biologic constituents (genes, proteins and metabolites organized in pathways and networks) deals with the known biologic knowledge in terms of pathway models. Data mining or reverse engineering approaches on the other hand deal with unknown knowledge that extract pattern and relationships from high-throughput data. A big challenge in cancer biology is how to better translate in-vitro innovations to the bed-side. Although still early, systems biology has the potential to revolutionize the understanding of cancer disease mechanisms. The development and application of cancer therapy can be facilitated by proving a systematic and integrative framework for incorporating data and outputting predictions. This would be feasible by connecting experimental data to computational approaches which can simulate known biologic systems for hypothesis testing. Hence combined systems biology computational and experimental approaches would enhance our understanding of human in-vitro tumorigenesis that can further aid in pharmaceutical drug development and creating a more rationale and predictive approach to the application of therapeutic strategies.

Molecular targeted therapies

Considerable chemotherapeutic advances have been made to target the cancer cells while sparing the normal ones. The classical ‘broad-spectrum’ cancer chemotherapy also affects the normal cells, thus adding to the therapy related toxicity. The field of molecular targeted therapy is still in its early stage, but a considerable response is evident when used with radiation. The main target in head and neck cancer has been COX-2, EGFR, VEGFR.

COX-1 is present in all the cells of the body while COX-2 is present predominantly in those cells showing inflammation or cancer. Since COX-2 mediates carcinogenesis, proliferation, angiogenesis, invasion, metastasis, and drug resistance, selective inhibitors of COX-2 are likely to show a beneficial effect. On the other hand prostaglandins are the potent protectors of normal tissues against radiation and therefore prostaglandin-inhibitors are likely to enhance the radiation response. The underlying mechanism for this increased radiosensitivity may be due to a combined effect of increase in intrinsic radioresponse, cell cycle redistribution from G1 to G2/M phase, induction of apoptosis, inhibition of growth factor regulation, anti-angiogenesis and immunostimulation. Hence the COX-2 inhibitors in combination with radiotherapy show a beneficial response by increasing anti-tumour activity, increased radiation sensitivity, but

sparing the normal tissues and hence the toxicity.

EGFR is a member of erbB receptor and tyrosine kinase family. Its increased expression is associated with increased radioresistance and increased cytostatic drug resistance. However no correlation has been seen between expression of EGFR and T-stage, N-stage or M-stage of tumour, but a positive association has been found with decreased survival and increased locoregional failure. The therapeutic strategies for targeting EGFR pathway include (1) Blockage of extracellular domain by C225, (2) Inhibition of intracellular kinase activity (Tyrosine kinase inhibitors) or down stream signaling pathways or through (3) Induction of truncated domain-negative mutant receptor (gene therapy). EGFR related 4 downstream pathways (RAS, PI3K, JAX, PLC γ) may be involved in tumour response through radiotherapy.

The antiangiogenesis mechanisms in molecular targeting have additional advantages in terms of (1) lack of drug resistance, (2) synergism with other treatment modalities and (3) lack of significant toxicity. Drugs like herceptin act indirectly by inhibiting VEGF synthesis while drugs like endostatin act directly by inhibiting VEGF, b-FGF, IL-8, PDGF. However there are limitations of antiangiogenic treatment in terms of (1) heterogeneity of endothelium, (2) delay in the onset of activity and potential tumour progression before response, (3) inability to eradicate microscopic disease when used alone. During radiation therapy VEGF increases and hence VEGF-inhibitors can be usefully combined with radiotherapy. Despite encouraging results a definite paradigm of combination of radiotherapy and anti-angiogenesis therapy has still to be worked out. Angiogenesis inhibitors may not solely function through perfusion mechanisms, but also by modulating matrix degradation and the paracrine functions; hence showing a synergistic response with radiotherapy.

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