HEAD AND NECK SQUAMOUS CELL CANCER: BIOLOGY (1)

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Abstract: This review is the first section of tumor biology pertaining to head and neck squamous cell carcinoma (SCCHN). It is intended to introduce the basic concepts of cancer biology to enhance the translational research. The basic tumour biology relates to the aberrations in the normal cell cycle, cell growth and cell death. The genetic aspects of cancer focus upon the roles of oncogenes, tumor suppressor genes and stability genes. The epigenetic mechanisms of the cancer relates to DNA methylation and histone acetylation. This review, discusses the basics of these concepts ..

Keywords: Squamous cell carcinoma of Head and Neck, Cancer Biology, cell growth

Recent developments in the field of cancer biology have revolutionized our understanding of carcinogenic mechanisms to ultimately focus on the targeted cancer therapies. With the constant improvement in the molecular techniques our knowledge of genetic and environmental interactions in carcinogenesis has considerably improved. In India, the head & neck surgeons (otolaryngologists) dealing with cancer are usually unaware of the overall concept of cancer biology. The concept of translational research does not exist amongst the majority of the teaching medical faculty across the country. The lack of a proper orientation to this concept in the regular teaching curriculum happens to be the main factor responsible for such a situation. We would like to introduce an overall broad concept of the cancer biology relevant to the head and neck cancer, to enable the treating surgeons to have a better understanding of this concept. This would further enable them to better collaborate with their basic science colleagues and to better generate appropriate hypothesis to be tested through translational research efforts.

Basic Tumour Biology:

Cell Cycle:

A normal resting cell in quiescent stage (G₀ phase) when 'plans' to divide undergoes 4 phases of cell cycle viz. G₁, S (synthesis), G₂ & M (mitosis). During G₁ or gap-phase, the cell prepares for the DNA synthesis that subsequently takes place in 'S' phase or synthesis phase. During G₂ phase the cell prepares for mitosis that subsequently occurs in M phase. However the majority of cells of cells in the body stay in resting state or the quiescent (G₀) phase. In order to proliferate the cells in G₀ phase get activated to enter G₁ phase under the influence of several growth factors provided in their environment such as EGF, IGF-I etc. The check points during transition of a cell from one to the other phase is regulated by cyclin family of cellular proteins. Cyclins, along with cyclin-dependent-kinases (COKs) are responsible for driving the cell through the cell cycle. The COKs are known to phosphorylate protein required for cell cycle progression. Hence proteins involved in driving the cell cycle, including the cyclins are frequently over-expressed in primary tumors. As the cell cycle progresses four major cyclins are sequentially produced viz. Cyclin-D, -E, -A, -B. In addition a family of proteins known as

inhibitors of kinases (INKs) counterbalance the cyclin's activation of COKs. Some of the known INKs are p15, p16, p18, p19. The cell cycle check-points are based upon pathways and feedback mechanisms ensuring that a phase of cell cycle does not begin until the preceding phase has been completed. The failure of check-points results in either apoptosis or genomic instability, which is important in the progression of uncontrolled proliferation. The requirement of growth factors to pass through G₁ phase is lost at the restriction (R) point, assumed to lie just before the cells start synthesizing DNA. Once beyond the R-point, the cells are committed to divide and they no longer require any extracellular growth factors. Hence R-point control is defective in cancer cells. Moreover a check point delay as induced by DNA damage at G₂-M check-point, gives time for DNA repair before cell undergoes mitosis. The G₂ check point is mainly controlled by cyclin B/CDK1 complex. It gradually accumulates during S-phase and early G₂, but is quickly released to allow the cell through G₂ phase. The storage and abrupt release of the complex is accomplished by the sequestration of CDK1 by inhibiting phosphorylation, that is under the influence of Wee1 (nuclear) and Myt1 (cytoplasmic) kinases. For mitosis to take place the cyclin B/CDK1 complex phosphorylation is reversed by Cdc25C & Cdc25p. If DNA damage is recognized, phosphorylation of Cdc25C takes place creating a new protein binding site. This bound to Cdc25p blocks the cyclin B/CDK1 complex by inhibiting its dephosphorylation. If however cell enters M-phase before repairing damaged DNA, then either apoptosis ensues or the resulting daughter cells carry mutations to result in cancerous proliferation. Check-point genes including p53 are involved in causing apoptosis as in BAX and other members of BCL-2 family. Mutations of half dozen or more cellular genes are required for tumor formation [1]. This number of events is very unlikely in normal cells, whose rate of mutation is approximately 10⁻⁷ per gene per cell division. Substances that modify check-point controls can change the rate of appearance of mutations and therefore the progression of cancer.

Cell growth:

The growth fraction or the proportion of cells in a tissue that are proliferating is around 30% in a typical head and neck cancer. It is well known that a normal cell has receptors on the cell membrane

that when stimulated by extraneous factors up-regulate the intracellular signaling to induce certain transduction factors which stimulate the particular gene to result in its expression (or cell division). Some of the extracellular factors that stimulate cell growth include: (1) peptide growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) which bind tyrosine kinase receptors located on the cell surface. (2) Cytokines such as growth hormone, interleukins, prolactins which bind to cell surface receptors other than kinases to result in signal transduction via interaction with separate tyrosine kinase molecules. (3) Some other growth factors can act through serpentine receptors that couple to intracellular pathways via heterotrimeric G proteins. (4) Steroid hormones such as estrogens which bind intracellular receptors also have mitotic activity. Unlike peptide growth factors, the steroid hormones do not initiate cytoplasmic signaling pathways but directly enter the nucleus to stimulate genes. In tumour cells the molecules regulating growth signaling pathways are often mutated resulting in constant 'on-signal' to the cell. These molecules can enhance growth (oncogenes) or inhibit it (tumour suppressor genes). In addition tumour cells often develop autocrine loops for growth and hence their growth becomes uncontrolled in a sort of 'positive feedback loop'.

In a typical example of EGFR a tyrosine kinase receptor, the binding of ligand on EGF receptor triggers the activation of its intrinsic tyrosine kinase activity and this in turn leads to phosphorylation of receptors. The intracellular domain of receptor becomes activated that in turn activates a complex cytoplasmic signaling to induce the relevant nuclear transcription factor. It is worth noting that phosphorylation activates p53 which deactivates pRB. The main tyrosine kinase growth receptors are RET, Trk, Met, Kit, IGF-1R, EGF-R, PDGF-R.

There are mainly five types of cell adhesion molecules: Cadherins are transmembrane proteins that possess five cadherin repeats which bind to other cadherins on adjacent cells. The integrins are heterodimers and consist of alpha and beta subunits. They interact with extracellular matrix for example the macromolecules fibronectin & laminin. The immunoglobulin-like adhesion molecules bind to integrins or to members of immunoglobulin family which may also be receptor themselves. The selectins contain a calcium dependent selectin domain that binds to carbohydrates. The proteoglycans are macromolecules possessing a small protein core with attached long chains of negatively charged glycosaminoglycans. These receptors combine with many other receptors but more importantly with extracellular matrix (ECM).

The ECM is a meshwork of glycoproteins, proteoglycans and glycosaminoglycans secreted by fibroblasts and provides support for cells to adhere and grow in tissues. The matrix itself is predominantly composed of type 4 collagen and makes up the connective tissue space between the cells. ECM is important in understanding the concept of carcinogenesis as it is invaded in the early stages.

Normally the cells attach to the matrix that signals the cells not to grow further. Moreover intercellular 'contact-inhibition' also does

not allow the cells to grow. During early carcinogenesis the cancer cells lose adhesion to the matrix as well as overcome contact-inhibition resulting in uncontrolled proliferation.

Cell death:

Cell can die by necrosis that usually happens in a disease, by cellular catastrophe during mitosis (eg following ionizing radiations), by paraptosis or being lost as in epithelia. Until recently necrosis was considered to be a random destructive process but now it has been shown that necrosis is organized by a set of genes and central to this process are proteases termed caspases. Apoptosis has been most widely studied in relation to cancer biology. Preprogrammed cell death or apoptosis is a critical step in cell differentiation, turnover and tissue homeostasis. This comprises of a precisely orchestrated series of steps triggered by physiologic stimuli that lead to membrane dissolution, breakdown of nuclear and cytosolic skeletons, chromosomal degradation and fragmentation of the nucleus. Apoptosis is currently considered to be a 3 step process: the 'initiation or step' signal (either extracellular or intracellular) triggers the apoptotic cascade; the 'execution' step is the actual program when multiple effector molecules cause the release of caspases to ultimately destroy the cell and the final step involves a series of morphological changes in the dying cell. Extracellular initiators of apoptotic cascade include several ligands that bind to 'death-receptors' such as Fas, TNF-R 1, TRAMP, TRAIL-R1, TRAIL-R2, & DR-6. The prototype model is Fas receptor. The Fas-ligand binds to the Fas-receptor that induces a signaling complex including intracellular binding of FADD (Fas associated death domain). This from procaspase-8 releases caspase-8 which initiates apoptosis by downstream effector caspase-3.

The intracellular initiators of apoptosis include the classical p53 gene response to external stress such as radiation, chemicals etc that induce DNA damage. The induction of apoptosis in this cascade appears to be through a downstream effector molecule Bax (potent pro-apoptotic molecule). Mitochondria play a key role in this pathway by releasing cytochrome-c' which in turn forms a complex with apoptosis inducing factor-1 (AIF-1), procaspase-9 and ATP. Within this complex procaspase-9 is activated to caspase-9 which then triggers caspase-3. The release of cytochrome-C is believed to be the key event in apoptosis and is regulated by the anti-apoptotic genes of the Bcl family (specially Bcl-2). Hence the mutation in p53 gene can result in the loss of function leading to survival of cells with damaged DNA. This in turn can lead to genomic instability and development of multiple genetic abnormalities that can enhance tumour progression. Significant correlation was also evident between Bax/Bcl-2 ratio and cyclin D1 levels and apoptosis. These observations suggest that apoptosis decreases as histological abnormality increases and this seems to be due to alterations in the level of apoptotic regulatory proteins.

Genetics of cancer:

Cancer is currently viewed as a genetic disease where genes involved in normal cellular functions are mutated or altered causing phenotypic changes of cancer. Three major classes of

such genes have been identified: (1) Oncogenes; (2) Tumour suppressor genes and (3) Stability genes.

The oncogenes in an inactivated state exist as proto-oncogenes that are important in normal intracellular signaling pathways. Proto-oncogenes may be rendered oncogenic in four ways: (1) de-regulation of expression, (2) gene mutation, (3) translocation, (4) amplification. The mutagenic events responsible for oncogenic conversion include retroviruses, inhaled carcinogens, dietary mutagens, low intake of antioxidants and free radical scavengers, ultraviolet & ionizing radiations, reduced immune-surveillance and DNA copying errors during cell division. Hence oncogenes activation results in increased expression of function either quantitative (increased in production of a normal product) or qualitative (production of abnormal product). Hence such activation may induce cellular proliferation and therefore tumour development. Typically the oncogenes require a single allelic change to render them tumorigenic and hence are transmitted in autosomal dominant fashion. The majority of genes are growth factor receptors (hst-1, int-2, EGFRJ erbB, c-erbB-2/Her 2, sis), intracellular signal transducers (ras, raf, stat-3), transcription factors (myc, fos, jun, c-myb), cell cycle regulators (Cyclin-D1) and those involved in apoptosis (bcl-2, BAX). Typical examples of such oncogenes involved in head and neck cancer include EGFR, STAT, & RAS. Various receptor such as receptor tyrosine kinases, activate RAS protein. The activated GTP-bound form of RAS exerts its effects through various target proteins such as mitogen-activated protein kinase and phosphatidylinositol-3-kinase cascades that are important in the activation of gene transcription and cell proliferation^[2]. When the growth signals are not required, the RAS proteins are de-activated to GDP-bound form, thus effectively "turning off" the cell growth processes. Therefore as a 'molecular switch', RAS exerts a significant effect on cell growth and differentiation pathways. As can be expected the mutated forms of RAS are likely to result in the constitutively activated protein causing uncontrolled cell growth typical of cancer.

Tumour suppressor genes are protective as they play a vital role in preventing normal cell from becoming malignant. 'Molecular policeman', a phrase quoted by Levine aptly describes these as a sort of molecular brakes preventing cells from undergoing uncontrolled growth. Most of them normally act as transcriptional regulators of the cell cycle, (RB I & p53) acting at check points to control entry into active proliferative states or apoptosis. They may also function as mismatch repair gene (MSH2, MSH6, MLR PMS I, PMS2), cell adhesion genes (APc' E-cadherin), DNA break repair genes (NBS 1,

fashion. The typical examples of tumour suppressor genes encountered in head and neck cancer are RB I, p53 and PTEN. Currently it is believed that even genetic heterozygosity for some of the tumour suppressor genes contributes to this transformation, a phenomenon labeled as haploinsufficiency.

Stability genes encode proteins that guard the cellular DNA by participating in nucleotide excision repair, DNA mismatch repair and chromosomal segregation in the mitosis. Although they themselves are not responsible for carcinogenesis but their dysfunction leads to accumulation of multiple genetic aberrations leading to cancer. The typical example of stability gene in HNSCC is FANCA that has shown to play a role in repair of double-stranded DNA breakage points^[3] as well as in genetic predisposition of HNSCC.

Molecular mechanisms of cancer:

There are several models hypothesized for carcinogenesis. (1) The Two-hit hypothesis of Knudson^[4] states that at least 2 events are necessary for carcinogenesis and that the cell with the first event must survive in the tissue long enough to sustain a second event. This was proposed in context with a tumour suppressor gene RB 1 where tumour occurrence was explained by the loss of both the alleles of this particular gene either successively (familial transmission) or simultaneously (sporadic occurrence). (2) Weinberg RA^[5] suggested that the activation of 2 or more oncogenes is required for Tumorigenesis and the right combination must be activated in the right context. For example Ras (cytoplasmic oncoprotein) cooperates with Myc (nuclear oncoprotein) to form tumours. (3) Boyd & Barrett^[6] suggested that tumour initiation is most likely a mutational event involving proto-oncogene such as Ras, while tumour promotion can be either mutational or an epigenetic change. This latter was defined as a series of qualitative and heritable changes in a sub-population of initiated cells, resulting^[1] in malignancy. (4) Vogelstein & Kinzler^[7] suggested a model of multistep carcinogenesis that includes both the activation of oncogenes and the loss of tumour suppressor genes. Accordingly they noted chromosomal losses at different steps of progression in colon cancer. This may be analogous to the various grades of pre-malignant conditions encountered in the head and neck and is discussed later. Although the precise order and number of events required for Tumorigenesis are not defined, six important steps seem to be necessary for cancer development [8,9]. These steps are: (1) Acquisition of autonomous proliferative signaling; (2) Inhibition of growth inhibitory signals; (3) evasion of apoptosis; (4) Immortalisation; (5) Angiogenesis; (6) Tissue invasion and metastasis. The description of these important steps follows in the second part of this article.

Molecular model of SCCN progression:

A multistep model of SCCN has been described by Hahn et al^[8] and Hanahan & Weinberg^[9]. A normal squamous mucosa under the influence of various carcinogens (such as tobacco and alcohol) up-regulate the extracellular factors such as EGFR resulting in squamous hyperplasia. Further by inactivation of p 16 and telomerase, the hyperplasia progresses to dysplasia. A few more

XPE, XPA, FANCA, FANB, cell cycle regulators (TSC2, INK4a), signal transducers (DPC4) and other transcriptional regulators (BRCA 1, BRCA2, Wt1). Hence when these genes are deactivated, via mutation, deletion or epigenetic silencing, the cell loses its inhibitory control to result in uncontrolled growth. For a cell to lose its inhibitory control over uncontrolled cell division, the loss of both alleles of tumour suppressor genes is necessary. Hence it is genetically transmitted in an autosomal recessive

genetic aberrations such as 3p deletion and p53 inactivation account for further progression to carcinoma-in-situ. Thereafter a multitude of genetic changes such as 4q, 5q, 8p, & 13q deletions result in frank invasive carcinoma. Such a localized carcinoma becomes more aggressive with ongoing other losses & metalloproteinase (over-expression to ultimately disseminate to distant sites). Hence it is important to characterize and stabilize the early genetic changes that may help in limiting cancer progression. Such a cancer chemoprevention program would include avoidance of carcinogens as well as prophylactic agents to counteract the existing carcinogenic influences.

Field Cancerization:

This concept was described by Slaughter et al^[10] to explain the development of multiple recurrences and second primary tumours in distinct areas of histologically normal mucosa of the upper aerodigestive tract. It has been seen that tumours of upper aerodigestive tract typically arise in the areas of dysplasia and are characteristically surrounded by dysplastic mucosa. Local recurrences and secondary primaries arise from the remnant dysplastic epithelium. Such dysplastic foci are thought to result from chronic insult by environmental mutagens (tobacco, alcohol), human papilloma virus infection etc. In accordance with the molecular progression model, a single abnormal cell with genetic alterations undergoes clonal expansion resulting in an area of mucosa with potential for carcinogenesis. The expansion of this clonal population gives rise to a wide area of unstable epithelium (single or multiple locations), which is more prone for malignant transformation when exposed to carcinogens.

Stem Cell biology of cancer:

Normally stem cells are intrinsically characterized by potential for (1) self-renewal, (2) extensive proliferation, (3) differentiation. However despite the clonal origin of many cancers, a notable heterogeneity is noted with solid tumours of head and neck. Solid tumour stem cells have been appreciated in breast and brain tumours and characteristically these breast tumours display a developmental hierarchy originating from low frequency tumour-initiating cells, which is phenotypically and functionally distinct from the bulk tumour [11]. Analogous to breast cancer stem cells, it is likely that stem cells will also be implicated in other solid tumours including head and neck. Theoretically the stem cells are hypothesized to cause distant metastasis, locoregional recurrence and incomplete response. Although solid tumour stem cell biology is in its infancy but certainly has a tremendous potential to revolutionize cancer biology.

Epigenetics of cancer:

The epigenetics relates to those heritable changes in gene expression that are not coded in the DNA sequence. It includes chemical modifications to DNA and its associated proteins to alter gene expression in CpG-rich domains without altering DNA sequence. Whereas genetic aberrations reflect the underlying base pair, the forms of epigenetic modification occurring in the human cells are known as DNA methylation and histone acetylation. Methylation of cytosine nucleotides directly switches off the gene

expression by preventing the binding of the transcription factors. This gene silencing (for example of tumor suppressor genes) is now recognized in the absence of any genetic change, suggesting a new model of bi-allelic inactivation^[12] involving hypermethylation, whereby one or both alleles might be affected by aberrant methylation at the gene promoter. Genes commonly found to be hypermethylated in cancer include tumor suppressor genes, metastasis related genes, DNA repair genes, hormone receptor genes and those inhibiting angiogenesis. In normal cells the pattern of DNA methylation is conserved following replication by a maintenance DNA methylase. On the other hand hypomethylation as evidenced by 10% reduction in genomic 5-methylcytosine content in premalignant and malignant lesions, presumably represents an overall increase in gene expression and cellular synthetic activity. The term methylotype has been used to signify the pattern of promoter hypermethylation. The hypomethylated genes in SCCN include p16, p15, RASSF1A, DAP-kinase, VHL, p73, E-cadherin, ABO, DCC, hMLH1, MGMT, p53, ATM, GSTP1, MINT-1, MINT-2, MINT-27, & MINT-31. In dysplastic lesions of the oral cavity, hypermethylation of p16 (INK4A) has been described.

Histone acetylation produces an 'open' structure of the histone-DNA complex resulting in inhibition of gene-silencing. The combination of both histone modification and acetylation is known as 'histone-code', and there occurs a significant cross talk between DNA methylation and histone code mediated gene silencing.

A most significant development in epigenetics has been the development of methylation-specific polymerase chain reaction (MSP) using bisulphate modification of DNA [13]. This precisely maps DNA methylation patterns across the entire genome and this analysis of circulating DNA can infer the elimination or persistence of tumor following treatment. The sources of free DNA have been shown to be serum (p16, MGMT, DAP-kinase), saliva/oral rinse (p16, p14, MGMT) and urine. Hence recognition of hypermethylation pattern in both the tumor tissue and circulating free DNA has a potential clinical application in early less invasive diagnosis and tumor surveillance.

At this point it is evident that the behavior of cancer cells is determined by the expression of their genome. Hence the pattern of gene expression may reveal many phenotypes of cancer including response to therapy and propensity of distant spread. In the past decades most of the clinical studies have correlated the level of expression on a few genes at the time of prognosis and in general the results have not been very satisfactory. Hence to overcome the limited predictive power of individual gene assessment, a multigene approach based on DNA microarray technology is supposed to enhance our understanding of carcinogenesis. By analyzing the pattern of multi gene expression a particular gene-signature is identified corresponding to cancer behavior. Such gene signatures after validation are likely to indicate the anticipated risk for progression and help in making cancer therapy choices. Despite the completion of human genome project our understanding is limited to only a quarter of these 30,000 genes. Hence high throughput technology is likely

REFERENCES:

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