INTRODUCTION

A variety of neoplasms with widely different natural histories can arise in the head and neck area, a relatively small body region essential for basic physiologic functions and social interactions. Depending on the location, size, and pattern of spread, head and neck tumors can cause various degrees of structural deformities and functional handicaps. Treatment of head and neck tumors can also induce additional disfigurements and malfunctions, worsening quality of life. Consequently, head and neck cancers are challenging to manage ***(Robert et al., 2012)***.

The incidence of head and neck squamous cell carcinoma (HNSCC) has been gradually increasing over the last 3 decades. It is the 5th leading cause of cancer by incidence and the 6th leading cause of cancer mortality in the world. Unfortunately, the majority of HNSCC patients present with advanced stage disease requiring multimodality therapy and even with combinations of intensive chemotherapy, radiotherapy, and surgery, cure rates are only 30% for advanced stage disease ***(Elizabeth et al., 2005).***

Despite advances in our knowledge of diagnostic and therapeutic methods for treatment of HNSCC, the survival rates for HNSCC have not markedly improved over the past decades. As such a deeper understanding of molecular carcinogenesis of

HNSCC is needed to further the development of more effective therapeutic applications ***(Gupta et al., 2009).***

The molecular tumor progression model states that tumors progress by activation of oncogenes and inactivation of tumor suppressor genes (TSGs), with each process producing a growth advantage for a clonal population of cells, CDKN2A (formerly designated p16; 9p21), TP53 (17p), and RB1 (13q) as candidate tumor suppressor genes, and CCND1 (cyclin D1 gene; 11q13) as a candidate proto-oncogene ***(Califano et al., 2003).***

About one third of histopathologically benign squamous hyperplasias contain a clonal population of cells with shared genetic anomalies characterizing head and neck cancer. Identification of such early events facilitates discovery of genetic alterations associated with further transformation and aggressive clinical behavior. With further validation, this knowledge will contribute greatly to the development of screening strategies ***(Ang et al., 2010).***

Over the last 15 years, advances in tumor biology have led to the discovery that many cancers, including HNSCC, appear to be supported by cells with stem-like properties. Studies in a wide variety of malignancies have demonstrated that only a distinct subpopulation of tumor cells, termed cancer stem cells (CSCs), contain the ability to undergo self-renewal and differentiation (properties of normal stem cells) and hence

have the ability to initiate tumorigenesis and support ongoing tumor growth, cancer stem cells have been increasingly shown to have an integral role in tumor initiation, disease progression, metastasis and treatment resistance ***(Marcus et al., 2O11).***

As with normal somatic stem cells, CSCs are defined by their ability to self-renew and to give rise to a heterogeneous population of tumor cells. This population of tumor cells consists of rapidly dividing cells (similar to the transient amplifying (TA) cell population in normal tissue) as well as additional CSCs and more differentiated tumor cells. In addition to their replicative capacity, CSCs , are also more resistant to the effects of cytotoxic chemotherapies and radiation damage ***(Al-Assar et al., 2009).***

CSCs were first experimentally defined in hematopoietic malignancies by ***John Dick*** and colleagues in 1994. Transplantation of a defined subpopulation of human acute myeloid leukemia (AML) cells (CD34HIGH CD38LOW) into immunodeficient mice was not only able to recapitulate AML but it was phenotypically and pathologically similar to the patient's original leukemia. In contrast, the remaining cell populations (CD34LOW and CD34HIGH CD38HIGH) failed to give rise to new leukemia cells ***(Dick et al., 1994).***

Since the identification of the leukemic stem cell, a number of investigators have identified CSCs in solid malignancies. In 2003; ***Michael Clarke and colleagues***

were the first to identify a CSC population in a solid tumor. A subpopulation of CD44HIGH CD24LOW breast cancer cells were able to recapitulate phenotypically heterogeneous breast cancers at very low limiting dilutions in mouse xenograft experiments. Since then a number of other groups have defined CSC populations in other epithelial malignancies including colorectal, prostate, lung, brain, and HNSCC ***(Al-Hajj et al., 2003).***

Several studies have demonstrated that drug or radiation treatment of tumor cells can enrich and maintain the CSC subpopulation in vitro and in vivo, suggesting that CSCs are responsible for tumor regeneration after conventional cancer treatments ***(Dylla et al., 2008).***

The identification of the cell population responsible for initiating tumorigenesis has significant implications for the prognosis and treatment of cancer. At present, cytotoxic chemotherapies target the rapidly cycling cells of the tumor and result in impressive reduction in tumor size, but leave the largely chemotherapy-resistant CSCs untouched, it is therefore theoretically possible that therapies which result in tumor cell death, as currently assayed, will not have any significant effect on the CSC and will therefore not result in long-term disease control or eradication ***(Hong et al., 2009).***

Such cancer stem or clonogenic cells tend to repopulate tumors during the course of radiotherapy and thus, by

extending a standard radiotherapy scheme over a period of 7 weeks may become a major cause of radiotherapy failure. It is postulated that quantification of CSCs could be useful in identifying subgroups of patients that would benefit from accelerated radiotherapy schemes or from biological interference targeting this cancer cell population ***(Koukourakis et al., 2012).***

Current experimental evidence suggests that CD44 expression characterizes a subset of cancer cells with stem-cell-like properties. This has been confirmed in several human tumors including squamous cell head-neck cancers ***(Xu et al., 2010).***

In 2007, ***Prince et al*** first identified a cellular subpopulation in head and neck tumors expressing the surface marker CD44 with stem-like characteristics; these cells were capable of reproducing when implanted into immunosuppressed mice. In the same year, ***Harper et al.*** studied the expression of CD44, CD29, and CD133 as presumed markers of CSCs in cell lines derived from head and neck tumors; they found that the greatest expression of CD44 correlated with increased clonogenicity.

CD44 is a type I transmembrane glycoprotein expressed in several cell types of mesenchymal and neuroectodermal origin. CD44 functions as a major adhesion molecule and in the cellular internalization of hyaluronic acid ***(Peterson et al., 2007).***

The bond between hyaluronic acid and the CD44 adhesion molecule may initiate a series of events that begin with modification of adhesion to the matrix and continue with activation of other molecules such as growth factors, degradation of the matrix, angiogenesis, permeation by blood vessels, and extravasation. All of these steps are necessary in the initiation of metastasis ***(Fang et al., 2011).***

CD44 is also involved in the transport of circulating lymphocytes to lymph nodes and in lymphocytic–epithelial interactions, through which it modulates lymphocyte adhesion and activation. These roles form the basis of the idea that CD44 plays an important role in lymph-node metastasis ***(Graham et al., 2007).***

The gene encoding CD44 comprises 20 exons; the first and the last five are constant, and the central ten are subjected to alternative splicing, thus constituting the variable region of the receptor. The most common isoform of the receptor is CD44 standard, About 30 receptor variants (CD44v) have been identified ***(Olsson et al., 2011).***

Recently, three different isoforms, CD44 v3, v6, and v10, have been shown to be associated with progression and metastasis of HNSCC. Increased CD44 v3 expression in primary tumors was associated with lymph node metastasis, while CD44 v10 expression was associated with distant metastasis and CD44 v6 expression was associated with

perineural spread. In cell culture, blockade of these CD44 isoforms with isoform-specific antibodies inhibited cellular proliferation, with the greatest inhibition seen with blockade of CD44 v6. Finally, increased expression of CD44 v6 and v10 was associated with shortened disease-free survival. These studies suggest that alteration in CSC phenotype through variation in CD44 isoform expression may alter the interaction of CSCs with the surrounding microenvironment. This may allow CSCs to more readily invade surrounding tissues or metastasize, there by promoting disease progression ***(Wang et al., 2009).***

Joshua et al have studied a lineage-CD44+ (Lin-CD44+) subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma, and they have observed a high frequency of Lin-CD44+ cells correlated with known poor prognostic factors such as advanced T classification and recurrence. In some cases, the overexpression of CD44v (v3 and v6) seems to reflect the cellular invasiveness and leads to increased aggressiveness of tumors in the head/neck ***(Allegra and Trapasso, 2012).***

In pathological conditions such as cancer, the extracytoplasmic domain of the CD44 receptor detaches and is released into biological fluids as a soluble fraction of the receptor CD44 sol, studing the levels of CD44 sol in the saliva of patients with tumors of the larynx starting from the assumption that in the normal upper aerodigestive tract, CD44

is expressed on the basal surface, whereas in the histologically dysplastic epithelium, CD44 is expressed in all layers of the mucosa in more than 90% of cases. This overexpression is also present in 90% of invasive head and neck tumor, the results show that high levels of CD44 sol in most patients with laryngeal carcinoma with high specificity compared with controls, and the highest levels of CD44 sol were observed in patients with advanced stages of disease ***(Allegra et al., 2012).***

Proliferative activity has been shown to be of prognostic significance for several malignancies, there is a clear correlation between proliferative activity and the biological behavior of cancer, which might have impact on the patient's prognosis and consequences for the individual therapy concept ***(Kuropkat et al., 1999).***

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0), makes it an excellent marker for determining the so-called growth fraction of a given cell population ***(Scholzen and Gerdes, 2000).***

A German study of the proliferative activity of 104 squamous cell carcinomas of the larynx (SCCL) was analyzed

retrospectively with the monoclonal antibody Ki-S11 which specifically detects the Ki-67 antigen.Results show that there was a statistically significant correlation ($p<0.05$) between histopathological grading, N-status and proliferative activity. There was also a significant difference for the 5-year survival between low and highly proliferating tumors. The patient group with low proliferating laryngeal cancer had a statistically ($p<0.05$) longer absolute and recurrence-free 5-years-survival time than patients with a highly proliferating cancer ***(Cordes et al., 2009).***

In this study we will investigate the correlation between the expression of CD44, proliferation index Ki-67 in head and neck squamous cell carcinoma treated with conventional chemoradiation, and the response to treatment, T-stage and N-stage.

AIM OF THE WORK

The aim of our study is to determine the rate of expression of Cancer Stem Cells marker CD44 & Ki-67 proliferation marker in tumor specimens of HNSCC, correlating data with the TNM staging, response rate to conventional chemoradiation therapy or definitive radiotherapy and the progression free survival of those patients, so to evaluate the prognostic value of CD44 and Ki-67 in HNSCC.

EPIDEMIOLOGY

Incidence and Mortality

Head and neck cancer (HNC) accounts for more than 550,000 cases annually worldwide. Males are affected significantly more than females with a ratio ranging from 2:1 to 4:1. Each year there are approximately 560,000 new cases of and 300,000 deaths due to HNC ***(Jemal et al, 2011).***

The highest incidences of HNC in the world are found in South Asia, and parts of central and southern Europe. The incidence trends are declining in the last decade in the Indian subcontinent, East Asia, Western Europe and the United States for men, but for women, it is generally stable ***(Boyle and Levine , 2008).***

Laryngeal cancer is the 11th most common cancer in men worldwide but is much less common in women. Men have been reported to have as much as 30 times the risk that women have for this disease. Older individuals are also at a higher risk for laryngeal cancer; the highest number of diagnoses is made in patient's age 60-74 years. Approximately 30-40% of laryngeal cancers originate in the supraglottis, while most occur in the glotti. Hypopharyngeal carcinoma represents approximately 7% of all cancers of the upper aerodigestive tract. The incidence of laryngeal cancer is 4-5 times that of hypopharyngeal cancer ***(Chu and Young, 2008).***

Despite aggressive treatment, only 35% to 55% of patients who present with locally advanced HNC cancer remain alive and free of disease 3 years after standard curative treatment. Thirty percent to 40% of patients develop locoregional recurrences, and distant metastases occur in 20% to 30%. Most recurrences appear quickly within 2 years of initial treatment and an additional 10% of patients will have evidence of distant metastases at the time of first presentation ***(Forastiere, 2003)*.**

Previous hospital-based studies from Egypt showed that HNC constitutes about 17-20% of all malignancies, In this first study describing the epidemiology of HNC in Egypt using data from the only population-based cancer registry, revealed higher incidence of HNC among males than females and higher incidence in urban than rural populations. Overall, the incidence of HNC was highest in the 70+ age group in both males and females ***(Attar, 2010)***.

Another epidemiology retrospective study in Egypt conducted at Head and neck surgery department Kasr-AlAini hospital during the period from Jan 2009 till Dec 2011. It was done on cases of laryngeal cancers revealed the mean age was (57.6 ± 10.5) ranging from (22 to 87) years old. Males were 93.9% while females were 6.1%. 62.4% were affected in the glottic and supraglottic regions, 20.7% transglottic regions ***(Tawab et al., 2014)*.**

Risk factors

Tobacco

Tobacco is the most important causative factor for head and neck Squamous cell carcinoma (HNSCC). Tobacco smoke, and in particular some of its components such as benzopyrene, can induce structural DNA damage. Host factors, including genetic variations of systems implicated in DNA damage correction or enzymes dedicated to metabolize these toxins, can also play a role in individual sensitivity to tobacco carcinogens. The induced damage may be repaired through the nucleotide excision repair (NER) or the base excision repair (BER) systems, the latter enabling the removal of a single base pair when a cytotoxic mutation is detected. Sequence variations in *NER*/*BER* genes could explain interindividual variations to tobacco toxins ***(Li et al., 2007).***

Numerous reports of gene polymorphisms of both repair systems are detailed in the literature. Some studies suggest that polymorphisms on the cytochrome P450 group, which render the enzyme more active, could explain why some individuals have a higher risk of developing HNSCC when exposed to tobacco. The same problem can occur when polymorphisms of detoxifying enzymes, such as glutathione *S*-transferase (GSP) or uridine diphosphate-glucuronosyl transferase (UDPGT), decrease their protective activity ***(Zafereo et al., 2009).***

In the International Agency for Research on Cancer (IARC) monograph, it was concluded that there was sufficient

evidence that the habit could cause not only lung cancer, but also cancers of the upper aerodigestive tract including the larynx, pharynx and upper esophagus ***(Menach et al., 2012).***

Alcohol

Alcohol increases the risk of upper aerodigestive tract cancer. The effect of alcohol is unknown, but it may be due to increased mucosal permability, liver damage and/or immune suppression. Chronic alcohol exposure results in the increased activation of carcinogens by cytochrome P450. On the other hand, the accumulation of acetaldehyde and other alcohol metabolites could affect gene transcription or have mutagenic effects ***(Seitz et al., 2007).***

Other risk factors have been reported: Plummer–Vinson syndrome, chronic infection with syphilis, long-term immunosuppression, poor oral hygiene and ill-fitting dentures. Rosenquist reported that poor oral hygiene, inadequate dental status and malfunctioning complete dentures were independent risk factors in oral and oropharyngeal squamous cell carcinoma ***(Rosenquist et al., 2005).***

Human papilloma virus (HPV)-related tumors

High-risk HPV is an important etiological factor for cancer of the oropharynx and is responsible for its increasing incidence. A meta-analysis showed that 26% of HNSCC contain HPV DNA and that more than 50% of all

oropharyngeal cancers are related to HPV infection, mainly HPV16 (95%) ***(Fakhry et al., 2006)***.

HPV and Laryngeal Cancer

The most common HPV type detected in laryngeal cancers is HPV16; HPV18 is the second most common HPV type, Duray demonstrated that the HPV16 viral load in laryngeal cancers (median 504 copies) was significantly higher than in benign lesions (median 37 copies). This supports the idea of active HPV "driver" infection, and suggested viral-mediated carcinogenesis ***(Duray et al., 2011)***.

Importantly, four studies examined the impact of HPV on the outcome of a total of 319 patients; 134 of which were HPVpositive. No association of HPV status with outcome was found ***(Morshed et al., 2008; Duray et al., 2011; Stephen et al., 2012 and Vlachtsis et al., 2005)***.

HPV and hypopharyngeal cancers

Hypopharyngeal squamous cell carcinoma in the pyriform sinus were more likely to be HPV-related than in other hypopharyngeal subsites. Also HPV infection status was related to the characteristic gross features of the tumors. Similar to HPV-related oropharyngeal cancers, HPV-positive pyriform sinus cancer showed a tendency for granulomatous and exophytic growth, whereas HPV-negative pyriform sinus cancer tended to demonstrate ulcerative or flat growth ***(Young-Hoon et al., 2013)***.