Published in final edited form as:
Growth Horm IGF Res. 2014 August ; 24(4): 137–141. doi:10.1016/j.ghir.2014.04.003.

Expression levels of insulin-like growth factor 1 and 2 in head and neck squamous cell carcinoma

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Abstract

Insulin-like growth factors (IGF) 1 and 2 are known as potential mitogens for normal and neoplastic cells. IGF2 is a main fetal growth factor while IGF1 is activated through growth hormone action during postnatal growth and development. However, there is strong evidence that activation of IGF2 by its E2F transcription factor 3 (*E2F3*) is present in different types of cancer. Also high levels of IGF1 strongly correlate with cancer development due to anti-apoptotic properties and enhancement of cancer cell differentiation, which can be attenuated by IGFBP3. Head and neck cancer is known as one of the six most common human cancers. The main risk factor for head and neck cancer is consumption of tobacco and alcohol as well as viral and bacterial infection by stimulation of chronic local inflammation. There is also a genetic basis for this form of cancer; however, the genetic markers are not yet established. In this study we investigated the levels of the expression of IGF2, *IGF1*, *E2F3* and *IGFBP3* in human cancers and healthy tissues surrounding the tumor obtained from each of 41 patients. Our study indicated that there is no alteration of the level of expression of *IGF2*, *E2F3* and *IGF1* in Head and neck squamous cell carcinoma (HNSCC) cases studied in selected experimental population, but there was evidence for upregulation of pro-apoptotic *IGFBP3* in cancer when comparing to healthy

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The Authors declare that there is no conflict of interest

tissue. These important findings indicate that insulin-growth factors are not directly associated with HNSCC showing some variability between patients and location of tumor. However, elevated level of *IGFBP3* suggests possible regulatory role of IGF signal by its binding protein in this type of tumors.

Keywords

oral cancer; *IGF1*; *IGF2*; survival

INTRODUCTION

Growth factors are strongly associated with cancer development. Insulin like growth factor 1 (IGF1) and IGF2 are major growth factors involved in development and growth. During fetal growth IGF2 plays the main role in development and mice with IGF2 disruption exhibit serious growth retardation at birth [1]. *IGF2* is an imprinted gene expressed only by the parental allele and alterations of *IGF2* imprinting are associated with childhood growth abnormalities [2, 3]. Biallelic expression of *IGF2* observed in Beckwith-Wiedenmann syndrome leads to overgrowth and is believed to predispose cancer development [4]. Moreover, studies with mice showed high expression of *IGF2* in different organs only during fetal life with dramatic down-regulation of its expression shortly after birth [5]. Importantly, the newest data indicate that different kinds of cancer are characterized by increased levels of *IGF2* in adults. While IGF2 acts as a main growth factor during prenatal development IGF1, mainly regulated by growth hormone, takes over as a main growth factor during post-natal development [6]. As GH stimulates the growth and development by activation of hepatic production and release to circulation of IGF1 there is also tissue-specific local IGF1 production which is believed to be GH independent. The release of IGF1 during growth can also increase the risk of developing or accelerating the growth of cancer. There is strong evidence that animals with GH/IGF1 deficiency live longer than wild-type controls [6] and at the same time are protected from cancer [6]. It was shown that treatment of dwarf rats with GH that stimulate IGF1 production increased mammary cancer risk [7]. This suggests that growth factors and the very specific regulation of them during prenatal and postnatal development can predispose individuals to the development of cancer in adulthood.

One of the sixth most common human cancers are oral cancers, commonly referred to as head and neck cancers [8]. There are several types of head and neck cancers, but about 90% are squamous cell carcinomas [9]. About 35,000 Americans are diagnosed with oral or pharyngeal cancer per year [9], and the number would be even higher (54,000) if laryngeal cancer was included. Worldwide the problem is much greater, with over 640,000 new cases being found each year. Head and neck carcinogenesis is a multistep process as a result of several genetic alterations. The dominant important risk factors for the development of head and neck cancer are the consumption of tobacco [10] and alcohol [11] [12]. Other factors include genetics, Human papillomavirus (HPV) infection [13] [14], as well as inflammation [15]. Another aspect of this devastating disease is its high death rate is particularly high. Approximately 13,500 deaths are reported every year in USA. The high death rate of oral

cancer is not because it is hard to discover or diagnose, but due to the cancer being neglected during patients' daily life. Head and neck cancers can affect physical and mental health, strongly affects patients' life quality and lifespan. Although improvement of early diagnosis of the cancer the best defense against this devastating disease[16] [17], regardless of early diagnosis and quick surgical intervention it is important to provide an appropriate strategy for treatment that includes chemotherapy and/or radiotherapy. Usually the therapy is adjusted individually based on the condition of the patients considering stage of tumor, overall health and age of patients. However, depending on characteristics of each individual case the treatment can be successful or may fail to cure the cancer. To determine a potential role of growth factors in head and neck cancer we investigated the levels of the expression of *IGF1*, *IGF2*, *E2F transcription factor 3* (*E2F3*) and *IGF1 binding protein 3* (*IGFBP-3*) in tumor and healthy tissues from 41 cancer patients subjected to surgical treatment.

MATERIALS AND METHODS

Patients

Forty-one patients with Head and neck squamous cell carcinoma (HNSCC) were included in the study with 28 males and 13 females. The average age of the participants was 59 years with the youngest patient being 37 years old and the oldest patient 70 years old. Patients diagnosed with HNSCC were subjected to surgical treatment in The Greater Poland Cancer Center. Material collected during the surgery included cancer tissue and, as a control, normal epithelium tissue was collected within the range of 2 cm distal from tumor margins from the same patient. Tumor grade of differentiation was evaluated following WHO criteria and the TNM classification was with accordance of International Union Against Cancer (UICC). Patients' clinical data is summarized in Table 1. All patients included in the study had not been treated with chemo- or radiotherapy prior to surgical intervention. The samples were divided into three groups according to the localization of tumor: larynx (n=25), oral cavity (n= 10) and pharynx (n= 5). The study was approved by the Institutional Review Board of University of Medical Sciences in Poznan and informed consent was obtained from all patients.

Exclusion criteria—According to the study protocol, patients with local recurrences and second primary tumor were excluded from experimental groups. Also patients with a previous history of chemo- or radiotherapy were excluded.

Quantitative RT-PCR

Tumour and normal tissue were collected during surgery and did not cause any additional risk for patient. Tissues were frozen in −80°C immediately after surgery was completed. Total RNA was extracted using TRI Reagent (Sigma) according to the manufacture's protocol. The concentration and purity of the eluted RNA samples were determined using the Take3 plate and the Epoch plate reader (BioTek). Complementary DNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed on the ABI 7900HT fast real-time PCR system, using Fast SYBR Green master mix with Rox (Applied Biosystems, Foster City, CA). Primers for IGF1 (PPH00167C), IGF2 (PPH00168B), and E2F3 (PPH00917F) were purchased from SAbiosciences. Primer

sequences for IGFBP-3 are: forward 5′-GCATGCTAAAGACAGCCAGC-3′, and reverse 5′-TCTCTACGGCAGGGACCATA-3′.

Statistical analysis

Analyses involved paired t-tests comparing gene expression values between tumor and healthy tissues. Given the exploratory nature of the analyses, we did not consider corrections for multiple comparisons. In order to determine if increases in expression levels of the genes in the tumors were associated with death or recurrence, we used Cox proportional hazards models [18] where death (or recurrence) was taken as the dependent variable with days until death (or recurrence) denoting survival times. 16 individuals died and 15 had recurrent tumors. Individuals who did not die (or who did not have recurrent tumors) were lost to follow-up after some period of time and were treated as censored observations. Gene expression levels in the normal and tumor tissue, as well as the differences between normal and tumor tissue, sex, age, and tumor grade were treated as predictors of death in the models. We fit models that considered gene expression levels for each gene independently as well as models that included all four genes as simultaneous predictors.

RESULTS

Gene expression analysis

The analysis of all patients indicated no significant difference in *IGF2* mRNA expression levels between healthy and tumor tissues (1.0±0.1909 and 1.365±0.3081 respectively; p=0.1585) (Figure 1A). Further, separation of patients according to the location of cancer also did not show any difference in the expression level of *IGF2* when comparing healthy tissue with cancer tissue in larynx (1.0±0.2651 and 1.184±0.3525 respectively; p=0.3392), oral cavity (1.0±0.3578 and 1.937±0.6596 respectively; p=0.1152) and pharynx (1.0±0.2604 and 0.5220±0.2344 respectively; p=0.1056) (Figure 1A, B, C). *E2F3* known as one of the stimulators of *IGF2* expression activator also did not differ between healthy and tumor tissue in whole group (1.0±0.2209 and 1.133±0.2868 respectively; p=0.3577)(Figure 1E). There was also no alteration of *E2F3* expression when analysing patients separately with cancer located in larynx (1.0±0.2867 and 1.465±0.5852 respectively; p=0.2396), oral cavity (1.0±0.3763 and 1.050±0.4959 respectively; p=0.4678) and pharynx (1.0±0.3183 and 1.6260±0.3603 respectively; p=0.2307) (Figure 1F, G, H). The expression level of *IGF1* also did not differ between healthy and cancer tissues in analysis performed on all 41 patients (1.0±0.1671 and 1.032±0.2218 respectively; p=0.4536)(Figure 2A). There were also no differences detected in larynx (1.0±0.2514 and 1.090±0.3600 respectively; p=0.4196), oral cavity (1.0±0.2308 and 1.097±0.3890 respectively; p=0.4163) and pharynx (1.0±0.2086 and 0.5920±0.3195 respectively; p=0.1581)(Figure 2B,C,D). Interestingly, there was evidence for *IGFBP3* mRNA level being upregulated in cancer when compared to healthy tissue (2.269±0.5522 and 1.0±0.1913 respectively; p=0.01131) (Figure 2E). Separate analysis of larynx cancer patients reflected the findings in whole experimental group indicating upregulation of *IGFBP3* in cancer tissue (2.519±0.6573 and 1.0±0.2230 respectively; p=0.0168) (Figure 2F). The same binding protein did not differ in oral (1.0±0.2979 and 1.822±0.7213; p=0.1531) and pharynx cancer groups (1.0±0.4573 and 0.8640±0.3770; p=0.4121) (Figure 2G, H).

We want to emphasize that for some analyses, our small sample size may have led us to be underpowered to detect an effect. For example, a power analysis using the t-test module in the G*power program [19] for detecting a difference in IGF2 between cancerous and normal pharynx tissue suggested that we would need 10 samples (we had 5) for the observed effect size assuming a power level of 0.80 with an assumed type I error rate of 0.05.

Survival analysis

Patients' post-treatment and survival—During patients follow-up period, there were 15 recurrences; the earliest recurrence was at 4 months after the operation and the latest at exactly 27 months after surgical intervention. During observation time there were 16 deaths, with the earliest at 2 months after operations the latest at 52 months after surgical treatment as presented by Kaplan-Meier (Figure 3).

Cox proportional hazards model analysis of *IGF1*, *IGF2*, *E2F3* and *IGFBP3*, when using the time until death from the time of the patients initial visit as the endpoint of interest (with age and sex as covariates), suggested that the difference in expression level between normal and tumor tissue for the *E2F3* gene was the only gene to have expression differences associated with survival times in models that assessed the effects of all 4 genes simultaneously (p=0.038; Supplemental Table 1). Decreased expression of the E2F3 gene in the tumor relative to normal tissue was associated with recurrence time. Tumor-normal expression differences in the IGF2 gene showed an association with recurrence times when analyzed simultaneously with the other the genes, suggesting decreased expression in tumor relative to normal was associated with recurrence time (p=0.041; Supplementary Table 1). This was the case in models that only considered each gene's effect in isolation of the others (p=0.044; Supplementary Table 1). We also found that elevated expression of the IGF1 gene in tumor tissue alone (i.e., not relative to normal tissue) was also associated with recurrence (p=0.0085; Supplementary Table 1). However, we must emphasize that our sample size was small with relatively few events (e.g., we had only 16 deaths and 15 recurrences among the 41 patients) and, as noted, given the exploratory nature of our analyses we did not control for multiple comparisons, so our results should be seen as highly preliminary.

DISCUSSION

There is a clear role of growth factors in cancer development and progression. *IGF1* and *IGF2* are known to decrease apoptosis and increase differentiation of cancer cells. Upon deregulation of growth factor expression, e.g. due to relaxation of *IGF2* imprinting and overexpression during postnatal development there, is an increased risk of cancer development. Importantly the expression of *IGF2* is regulated by *E2F3*. After birth *E2F3* is strongly supressed which also downregulate the expression of *IGF2* [20]. However, it was previously published that several types of cancer are characterized by elevated levels of *IGF2* and *E2F3* [20]. In contrast to observation in prostate, urinary bladder or Wilm's tumor [20] our study of HNSCC cancer patients did not show any difference in *IGF2* mRNA expression levels when cancer and healthy tissues from the same individuals were compared. This finding indicate that the expression of *E2F3* and *IGF2* in HNSCC does not

play the same part in head and neck cancer biology as it has been indicated in other kinds of cancers characterized by elevated levels of *E2F3* and *IGF2* [20].

Similarly, the level of *IGF1*, the main postnatal growth factor, was also not altered in oral cancer tissues; however, there was a surprising increase of *IGFBP3* mRNA in tumors. There is some evidence that IGFBP3 regulates the action of IGF1. Usually individuals with lower IGF1 and higher IGFBP3 would have lower risk of cancer [21], which would suggest that interaction of IGFBP3 with IGF1 would block the anti-apoptotic action if IGF1. However, at the same time binding of IGFBP3 to IGF1 can increase the half-life of IGF1 which could be rather negative for cancer patients. Moreover, the mRNA expression level in tumor and healthy tissue does not allow us to present clear conclusions without future studies of serum protein levels, binding assays and completing more mechanistic experiments involving the regulation of IGF1 by IGFBP3 in HNSCC. However, as serum levels of IGF1 and IGFBP3 can be associated with different types of cancer including prostate, colon/rectum, breast, lung, stomach [22–26] and even childhood leukaemias its role is still not well established in HNSCC. We would expect that high levels of serum IGF1 would be also associated with head and neck cancers, however, Brady et al. showed that circulating levels of both IGF1 and IGFBP3 protein is decreased in head and neck cancer patients [27]. On the other hand there is an evidence that IGF1 therepy for head and neck cancer undergoing radiation therapy may help with preservation and restoration of silvary gland function after radiation therapeutical approach [28]. Yet, the study of oral cancer cell line showed that there is positive association between autocrine production of IGF2 together with overexpression of *IGF1-R* and enhanced proliferation of oral cancer cells [29]. This could suggest that the lack of association between *IGF1/IGF2* and HNSCC in selected population appears surprising; however, it could suggest that aside from local mRNA expression the circulating level of growth factors could be also important and have different role in HNSCC cancer development, etiology and during treatment which should be further investigated and correlated with protein levels of IGF1 and IGF2 in serum and tumor tissue. Additional complication for the study can also come from the cause of HNSCC such as genetic, environmental or viral infection as well as location. Our data indicated that there is a tendency towards upregulation of *IGF2* while pulling all locations as well in larynx and oral cavity only. Interestingly, there was opposite trend indicating almost 50% down regulation of *IGF2* in patients with tumor localized in pharynx. Symilar trend was observed for the expression of *IGF1* and *E2F3 mRNA in pharynx which could suggest differential biology and mechanism involved in HNSCC in this particular location.*

In summary, in studied 41 patient affected by head and neck cancer we found that HNSCC are not associated with upregulated expression of IGF1 or IGF2 and observed elevation of IGFBP3 mRNA suggests that activation of IGF1 signal may be affected in these types of cancer cells. However, a suggestive relationship between *E2F3* and survival suggests that a greater expression difference of *E2F3* between normal and tumor tissue will possibly predict shorter survival of the patient affected by HNSCC.

Acknowledgments

Research reported in this publication was supported by *National Institute on Aging* of the National Institutes of Health under award number R01AG032290, Polish National Science Centre N N403 186934 and NJS is funded in part by NIH grant 5 UL1 RR025774.

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Highlights

- Insulin growth factors are not directly associated with HNSCC
- The expression of E3F3 suggest relationship with the prediction of survival of HNSCC patients

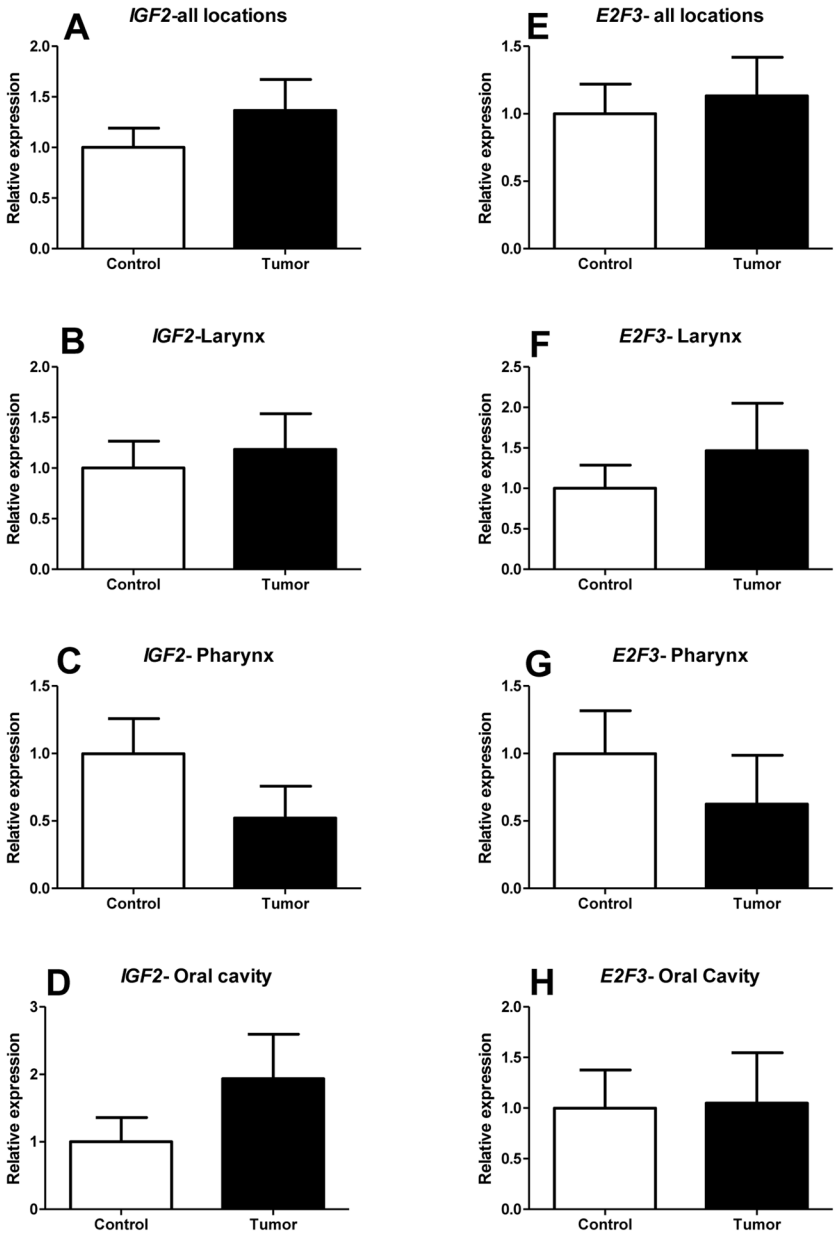

Figure 1.

The levels of relative expression of insulin-like growth factor 2 (*IGF2*) and E2F transcription factor 3 (*E2F3*) genes in Head and neck squamous cell carcinoma (HNSCC) and healthy tissues from the same patients. Means ± SEM.

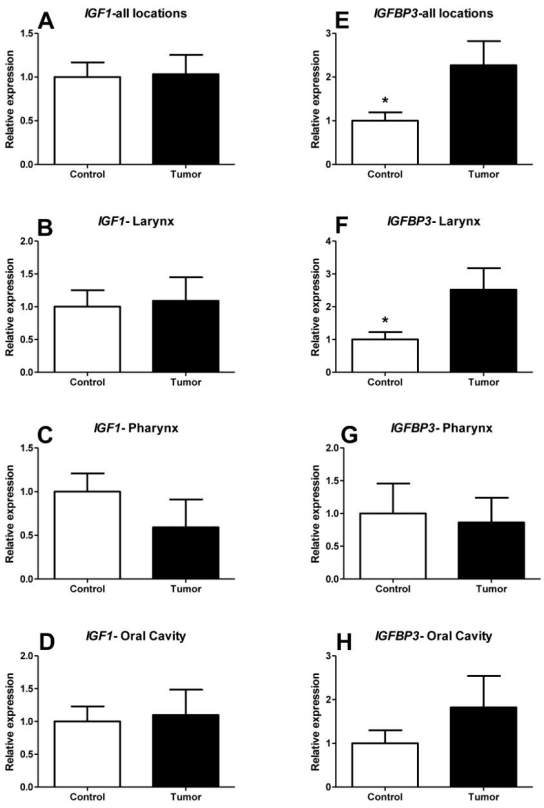

Figure 2.

The levels of relative expression of insulin-like growth factor 1 (*IGF1*) and IGF binding protein 3 (*IGFBP3*) genes in Head and neck squamous cell carcinoma (HNSCC) and healthy tissues from the same patients. (*) represent significant difference ($P<0.05$)

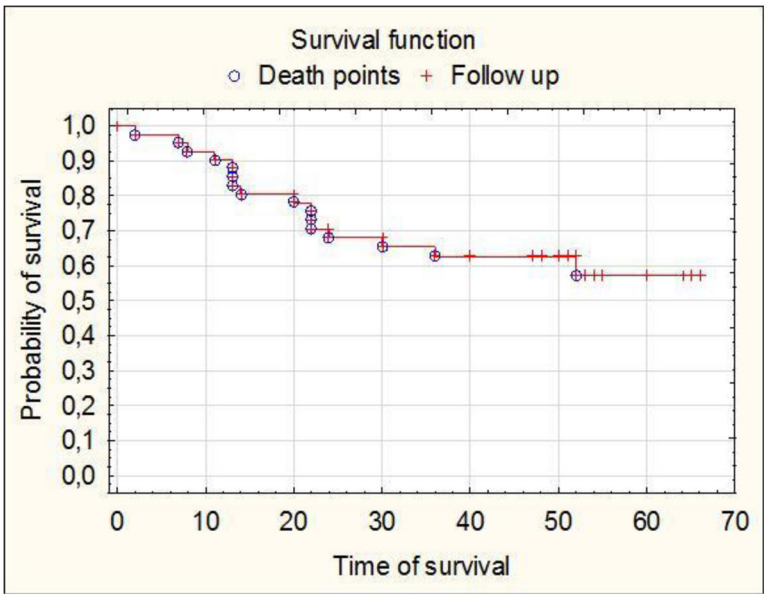

Figure 3.
Kaplan-Meier survival curves representing patients' post-surgical follow-up.

Table 1

Summary of patients' clinical data.

Gender	female	13			
	male	28			
Median age at diagnosis (y)		59			
Anatomic site		**larynx**	**oral cavity**	**pharynx**	**other**
Total number		25	10	5	1
Tumor grade	Low-grade (I)	0	2	1	0
	Intermediate-grade (II)	20	6	4	0
	High-grade (III)	3	2	0	1
Stage	Low (I, II)	5	3	1	0
	High (III, IV)	18	7	4	1
M status	positive	1	0	0	0
	negative	24	10	5	1
N status	Low (0, I)	25	10	5	1
	High (II, III)	0	0	0	0