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# Loss of imprinting and promoter usage of the *IGF2* in laryngeal squamous cell carcinoma

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## **Abstract**

The gene for insulin-like growth factor two, *IGF2* is maternally imprinted. Fifteen heterozygous samples were analyzed for the *IGF2* imprinting status and promoter usage. *IGF2* LOI was detected in four non-tumorous tissues and in six laryngeal squamous cell carcinoma (LSCC) tumors. There was no clear pattern of specific promoter activity in LSCC tumors and the adjacent normal tissues. P1 promoter usage was active in eight LSCCs, among them four with LOI. As it was activated in four tumors with maintenance of imprinting (MOI) and four non-tumors, we concluded that P1 promoter is not exclusively connected with *IGF2* LOI in LSCC.

Keywords: *IGF2*; Laryngeal squamous cell carcinoma; Imprinting; Promoter usage

## 1. Introduction

Genomic imprinting is a parental origin-specific chromosomal modification which causes differential expression of maternal and paternal alleles. IGF2 is a mitogenic peptide which exerts its bioactivity through binding to the type I IGF receptor. The gene encoding IGF2 (*IGF2*) is maternally imprinted with only paternal allele being transcribed. It is overexpressed in a wide variety of neoplasms and it is thought to serve as autocrine tumor enhancer [1]. Using SAGE technology, Zhang et al. showed that *IGF2* transcripts were 73 times more abundant in colorectal cancer tissue than in non-tumorous tissue [2].

The *IGF2* spans 30 kb of chromosomal DNA and is located on chromosome 11 (11p15.5). It consists of nine exons and only exons 7, 8 and the 5' part of exon 9-encode the IGF2 prepro-protein. Exons 1, 4, 5 and 6 are preceded by separate promoters (P1-P4) that give rise to a family of *IGF2* mRNA species of different length. The activity of these promoters is regulated in a tissue- and developmental-specific manner. In prenatal and early postnatal liver and chondrocytes, *IGF2* transcripts from the P1 promoter are always derived from both parental alleles, whereas transcripts from the P2-P4 promoters are always active from one parental allele [3, 4]. In adult liver, P1 promoter activity is increased. It is equally active on both alleles and encompasses the entire *IGF2* transcriptional unit (including P2-P4 promoter sequences) [4]. Loss of *IGF2* imprinting (*IGF2* LOI) could then result from promoter switching if transcription from the imprinted promoters P2-P4 declines and transcription from the non-imprinted promoter P1 increases.

It is not clear whether the usage of the *IGF2* promoters changes with the progression of some malignant tumors. To date, it has not been clarified whether promoter usage is altered in laryngeal squamous cell carcinoma (LSCC) as compared with normal laryngeal tissue.

## 2. Materials and methods

### 2.1. Tissue samples

Tissue samples were obtained from the University Hospital “Sestre milosrdnice”, Zagreb, Croatia, during surgical procedures. All patients were male, ages 46-83, with diagnosed laryngeal squamous cell carcinoma. TNM staging was performed at the Department of Pathology “Ljudevit Jurak”. Informed consent was signed by all patients. The tissues were quickly frozen in liquid nitrogen and stored at -80°C until analysis.

### 2.2. Genomic DNA and total RNA extraction

Approximately 1 cm<sup>3</sup> of frozen tissue was used for the extraction of genomic DNA by the phenol-chloroform method [5]. For total RNA extraction, tissue was homogenized in RNA-Bee reagent (Biogenesis Ltd. Poole, England) and total RNA was extracted according to the manufacturer’s instructions. It was further purified by using a RNeasy<sup>®</sup> Mini kit (Qiagen, USA). RNA was treated on column with DNase (Qiagen, RNase-free DNase set) in order to remove traces of contaminating DNA. RNA integrity was determined by electrophoresis on a 1% agarose gel.

### 2.3. Identification of genomic IGF2 *ApaI* polymorphism

Genomic DNA was isolated from non-tumorous tissues and analyzed for heterozygosity for the *ApaI* restriction site in the *IGF2*. For this purpose, primers *ApaIF*: 5`CTTGGACTTTGAAGTCAAATTGG 3` and *ApaIR*: 5`ATCGTTGTTGGTCTGACCGAGGAG 3` were used for the amplification of 173 bp long segment of exon 9. The 25 µl reaction mixture contained: dNTPs (50 µM each), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 8 pmol of each primer and 0.25 U of rTaq polymerase (TaKaRa). A total of 38 cycles was performed. One cycle consisted of denaturation at 95°C for 30s, annealing at 57°C for 30s and primer extension at 72°C for 30s (+ 1s in every subsequent cycle). The PCR product was digested overnight with 10 U of *ApaI* (Roche), yielding 173 bp fragment (homozygote - uncut),

108 and 65 bp fragments (homozygote - cut) or 173, 108 and 65 bp (heterozygote - one allele cut, another uncut). Digested PCR products were electrophoresed through 10% polyacrilamide gel and visualized by silver staining [6].

#### *2.4. Analysis of IGF2 expression and imprinting status*

One microgram of total RNA was reversely transcribed using 0.5 µg/µl oligo(dT)<sub>18</sub> (New England BioLabs), 0.5 mM dNTPs, 20 U of RNase inhibitor (Roche), 1x incubation buffer (supplied with M-MuLV enzyme: 75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiotreitol; pH 8.3) and 40 U of M-MuLV reverse transcriptase (New England Biolabs) in total volume of 20 µl. RT PCR cycle consisted of pre-incubation of RNA with oligo(dT)<sub>18</sub> at 70°C for 10 minutes and incubation at 37°C for one hour.

The quality of cDNA was checked by amplifying part of the housekeeping gene, *GAPDH*, with primers GAPDH1: 5' AACGGATTTGGTCGTATTGGGC 3' which is located on border between the first and the second exon and GAPDH2: 5' AGGGATGATGTTCTGGAGAGCC 3' which is located in exon seven. An additional check, this time for DNA contamination, was made with primers GAPDH2 and GAPDH3: 5'AAGCTGACTCAGCCCGCAAAGG3' which is complementary to DNA sequence in intron five. The 25 µl reaction mixture contained dNTPs (50 µM each), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 6 pmol of each primer and 0.25 U of rTaq polymerase (TaKaRa). Forty-two cycles were performed with the annealing temperature of 56°C. The PCR products (sizes 605 and 642 bp, respectively) were electrophoresed through 1% agarose gel and stained by ethidium bromide (0.5 µg/ml).

Successfully transcribed cDNA, free of contaminating genomic DNA, was then amplified with primers ApaIF and ApaIR. The PCR product was 173 bp long. Amplicons were digested with *ApaI*, as already described, and run through 10% polyacrilamide gel. Bands were visualized by silver staining.

#### *2.5. Promoter specific expression of IGF2*

Promoter specific expression was analyzed only on samples showing *IGF2* expression (mono- or biallelic). Promoter-specific sense primers were used to evaluate transcription of *IGF2* from four promoters:

P1S: 5' CGAATTCTGGGCACCAGTGA CTCCCCG 3' located in exon two (GenBank accession number X03423); P2S: 5'ACCGGGCATTGCCCCCAGTCTCC3' located in exon four (GenBank accession number X03562), P3S: 5' CGTCGCACATTCGGCCCCCGCGACT 3' located in exon five (GenBank accession number X03562) and P4: 5' TCCTCCTCCTCCTGCCCCAGCG 3' located in exon six (GenBank accession number X03562). The common antisense primer was PR: 5' CAAGAAGGTG AGAAGCACCAGCAT 3' [7] located in exon seven (GenBank accession number: X03562). The sizes of PCR products were 376 bp, 254 bp, 186 bp and 134 bp respectively. Touchdown PCR was performed as follows: in the first 13 cycles annealing temperature was getting lower for 1°C starting from 68°C, followed by 25 cycles with the annealing temperature at 55°C. PCR products were loaded on 2% agarose gel and stained with ethidium bromide.



### 3. Results

We have analyzed 15 LSCC tumors and comparable normal tissues to determine *IGF2* imprinting status and promoter usage. In most specimens (7 out of 12), the tumorous and non-tumorous tissues did not have the same pattern of promoter usage (Table 1). The most frequently active promoter in non-tumorous tissues (denominated NT) was P3 (10 out of 12 samples), followed by P4 promoter (9 out of 12 samples). Therefore, we concluded that, in the majority of normal laryngeal tissues, P3 and P4 promoters directed transcription of *IGF2*. Unexpectedly, P1 promoter was active in four non-tumorous samples: two with retained *IGF2* imprinting (NT3 and NT8) (Fig.1) but also in two specimens showing biallelic expression of *IGF2* (NT11 and NT14). In sample NT8, it was the only active promoter. However, in sample NT11, P3 promoter was also activated and, in samples NT3 and NT14, transcription was directed from all three of them. P3 promoter was active alone in sample NT2. P4 promoter was active alone in sample NT5. A combination of P3 and P4 promoters occurred in 50% of the samples (NT1, NT4, NT6, NT7, NT13 and NT15).

In tumor specimens, the most frequently used promoter was P4 (11/15), followed by P3 (10/15) and P1 promoters (8/15). P1 promoter was used in four out of six tumors that expressed *IGF2* biallelically (T9, T11, T12 and T14). It was also active in other four tumors (T1, T2, T6 and T10) which maintained *IGF2* imprinting. In three samples, P1 promoter was active alone and others were shut down (T1, T2 and T6). In more than a half of the samples, P1 promoter was used in combination with P3 (T11), P4 (T10) or both of them (T9, T12 and T14). P3 promoter was not active alone in either tumor specimen. P4 promoter was active alone in one sample (T7) while a combination of both promoters occurred in six tumors (T3, T4, T5, T8, T13 and T15).

We did not detect expression from P2 promoter at all.

#### 4. Discussion

We noticed biallelic expression of *IGF2* in four non-tumorous tissues. Our results are consistent with results from Cui et al., who found *IGF2* LOI in both the normal tissue and tumor of patients with colorectal cancer (CRC) [8]. Also, the odds of LOI in normal tissue of individuals with a family history of CRC, were 5.15 times that of individuals without it. The odds of LOI in participants with a personal history of CRC were 4.72 times that of participants without it [9]. These data suggest that loss of *IGF2* imprinting leads to an oncogenic diathesis that enhances the risk of neoplastic transformation [10]. Hence, we consider non-tumorous tissues with biallelic *IGF2* expression (although morphologically unchanged) epigenetically changed. This phenomenon might be the first step in the neoplastic transformation on this model as proposed for the CRC [9].

P1 promoter was active in non-tumorous tissue in specimens with monoallelic (NT3 and NT8) and biallelic *IGF2* expression (NT11 and NT14). Activity and imprinting of the *IGF2* P1 promoter apparently occurs in a tissue-specific manner since it is not active in cervical tissue [11], benign bladder, breast and prostate tissue [12]. Its activity was shown in pons [13] and in adult skeletal muscle tissue [14] where *IGF2* is expressed monoallelically. In addition, in adult human liver and chondrocytes [3, 4], P1 governs biallelic transcription.

We detected the activity of P1 promoter in eight laryngeal tumor samples. Among them were four with LOI (T9, T11, T12 and T14). We concluded that the activity of P1 promoter is upregulated in LSCC tumors but apparently is not causing LOI because only half of LOI tumors expressed *IGF2* from P1. Relationship between P1 promoter usage and *IGF2* LOI apparently differs between types of tumors. For example, in Wilms' tumors P1 transcripts were present in all LOI tumors but also in some MOI tumors [15]. In cervical carcinoma tissues P1 promoter (sometime in combination with P3) was used only in carcinoma tissues in which *IGF2* LOI occurred [11]. In rhabdomyosarcoma tumors expression from P1 promoter was detected in 20% of *IGF2* LOI tumors and in 30% of MOI tumors [16]. Unexpectedly, in three LSCC tumors (T1, T2 and T6) the *IGF2* was imprinted (only allele "a" was transcribed) from the P1 promoter. Although this result does not correlate with the well known hypothesis: "P1 active – biallelic expression present", there are results in the literature that are similar to ours [15, 16]. One possible explanation is that there

are tissues and developmental specific factors which can modulate the imprinting signal responsible for the differential *IGF2* allelic expression [15], even more because we have shown this phenomenon in one non-tumorous tissue (NT8). It would be of great interest to study the methylation pattern in these samples by the bisulfite sequencing method.

Our results are also contrary to the finding of Issa et al. who demonstrated a switch, caused by the spreading of methylation from maternal to paternal *IGF2* P2-P4 promoters associated CpG island, from P2-P4 promoters to P1 promoter during carcinogenesis [17].

In non-tumorous laryngeal samples, we detected the activity of different promoters at the same time. We do not know which promoter governed biallelic expression in samples with *IGF2* LOI because previous observations demonstrated that P3 promoter could also direct biallelic expression [12, 15, 18, 19].

Expression from P2 promoter was not found at all. P2 promoter is active during fetal life [20] and the level of mRNA directed from P2 is quite low in all tissues and cell lines. One might ask what its physiological role in adult tissues is [21]. The mechanism of regulation of P2 promoter activity is still unclear [20]. Von Horn et al. showed that acute growth hormone (GH) treatment doubles transcription from P2 promoter in adult human liver [21]. Most of our patients were older than 60 years, so an explanation for no detection of P2 mRNA could be the silencing of P2 promoter during aging.

Finding that in normal laryngeal tissues P3 and P4 were the most active promoters is in line with the results of Mineo et al. who demonstrated that, by using semi-quantitative RT-PCR, expression from P3 and P4 promoters predominates in human benign bladder, prostate and breast tissue [12].

Transcription from P3 promoter is also upregulated in such human tumors as: hepatoblastoma [22], Wilms' tumor [23], breast, prostate and bladder cancers [12]. The proximal region of P3 promoter contains several methylation-regulated transcription factor binding sites, important for the regulation of its activity [24]. In LSCC samples, P3 activity, when present (in 10 out of 15 samples), was always joined with the activity of P4 (T3, T4, T5, T8, T13 and T15), P1 (T11) or P1 and P4 promoters (T9, T12 and T14). When comparing three tumorous and non-tumorous tissues retaining imprinting (samples 1, 2 and 6), transcription from P3 promoter,

present in non-tumorous tissue, was replaced with P1 promoter activation in tumorous tissues. The reason for this switch in laryngeal tumors could be hypermethylation.

In conclusion, the usage of promoters differs in laryngeal squamous cell carcinomas when compared with non-tumorous tissues. There is no clear pattern of *IGF2* promoter usage either in laryngeal tumors or in adjacent normal tissues, although P1 was more frequently active in tumors than in normal tissue. Our study also demonstrates that *IGF2* promoter usage is highly variable in malignant and adjacent normal laryngeal tissue and that P1 promoter usage is not responsible for *IGF2* LOI in LSCC.

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## Tables

**Table 1:** Results of promoter usage in tumor and matched non-tumorous tissue

<i>sample</i>	<i>IGF2 expression</i>	<i>P1</i>	<i>P3</i>	<i>P4</i>
NT1	allele a		active	active
T1	allele a	active		
NT2	allele a		active	
T2	allele a	active		
NT3	allele b	active	active	active
T3	allele b		active	active
NT4	allele a		active	active
T4	allele a		active	active
NT5	allele a			active
T5	allele a		active	active
NT6	allele a		active	active
T6	allele a	active		
NT7	allele a		active	active
T7	allele a			active
NT8	allele a	active		
T8	allele a		active	active
NT9	no expression			
T9	alleles a and b	active	active	active
NT10	no expression			
T10	allele a	active		active
NT11	alleles a and b	active	active	
T11	alleles a and b	active	active	
NT12	no expression			
T12	alleles a and b	active	active	active
NT13	alleles a and b		active	active
T13	alleles a and b		active	active
NT14	alleles a and b	active	active	active
T14	alleles a and b	active	active	active
NT15	alleles a and b		active	active
T15	alleles a and b		active	active

## Figure legend

**Fig. 1.** Lanes 1- 4: Promoter usage in non-tumorous tissue NT3 where promoters P1 (376 bp, lane 1), P3 (186 bp, lane 3) and P4 (134 bp, lane 4) direct monoallelic transcription of *IGF2*. Lanes 6 - 9: Promoter usage in tumor T3 (monoallelic expression), promoter P1 is silenced (lane 6) and promoters P3 (186 bp, lane 8) and P4 (134 bp, lane 9) are active. The expression from promoter P2 (lanes 2 and 7) was not detected. Lane 10: *Hae*III digest of  $\Phi$ X174 DNA (New England BioLabs); lane 5: marker V (Roche).

