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EXPRESSION OF SEXUAL HORMONES RECEPTORS IN ORAL SQUAMOUS CELL CARCINOMA

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Sexual hormones play an important role in expression of genes involved in a wide variety of biological and neoplastic processes. The information on Estrogen Receptors (ER) expression in non-target tissues is very few and, in particular, the studies in head and neck tumors are still controversial. Recent studies analyzed the role of Tamoxifen (TAM) on Oral Squamous Cell Carcinoma (OSCC) lines in relation to the presence/absence of ER. The purpose of the present study was to evaluate the expression of sexual hormones receptors mRNAs, in particular Estrogen Receptor alpha (ERα) and Androgen Receptor (AR) mRNA in OSCC tissues. The study group comprised 20 samples of OSCC, harvested from 20 otherwise healthy subjects (14 males and 6 females, mean age 58.2y, range 38-74). The control group was formed by 20 samples of normal mucosa harvested around the margins of the specimens (at least 1 cm from the lesion margins). Estrogens Receptor alpha (Era) and Androgen Receptor (AR) mRNA expressions were analyzed by RT-PCR carried out on total RNAs extracted from both cancerous and healthy tissues. Obtained data were evaluated by Shapiro-Walk normality test and compared by Student's t test. Results with $p<0.05$ were considered statistically significant. AR transcripts were less expressed in OSCC specimens than in healthy tissues, while levels of ERα transcripts significantly increased in tumor samples. These preliminary data show different expression patterns of AR and ERα mRNAs in malignant tissues of oral mucosa and could suggest an involvement of these sexual hormones in oral cancer.

Five percent of all tumors occur in the head and neck region and approximately half of those occur specifically in the oral cavity. Among these malignancies, the most frequently encountered is undoubtedly the Oral Squamous Cell Carcinoma (OSCC) accounting for more than 90% of cases (1) . Its prevalence increases with age and with abuse of risk factors such as alcohol and tobacco (1). Oral cancer shows well-established multi-phasic and multi-factorial dynamics. It is well known that the multi-step model of oral carcinogenesis requires the step-wise transition from pre-malignant conditions to the tumor phenotype (2). A variety of genomic alterations accumulates and potentiates this transition gradually promoting the progression from normal mucosa, to dysplasia, until to carcinoma in situ and advanced cancer (2).

Despite great financial and scientific efforts, the percentage of long-term survival in patients with OSCC is still low, especially when a definitive surgery is not applicable, because low efficacy of anticancer drugs. Thus, a better understanding of the molecular profile of oral carcinogenetic processes should facilitate the development of more efficient targeted therapies (3).

The steroid hormones play an important role in expression of genes involved in a wide variety of biological and neoplastic processes. In particular, the role of the Estrogen Receptor (ER) and estrogen-regulated protein in various normal and pathological tissues (such as breast cancer) has been widely studied (4) and hormonal

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treatment (i.e. non-steroidal Selective Estrogen Receptor Modulator - SERM) has been proposed and successively applied. Therefore, the information on ER expression in non-target tissues are very few and, in particular, the studies in head and neck tumors are still controversial (5). To date, only immunohistochemical studies have investigated on the presence of steroid receptors in head and neck tumors (6). In fact, ER and Progesterone Receptor (PgR) proteins in salivary gland adenoid cystic carcinoma have been identified and the possible use of endocrine therapy as a modality of treatment has been suggested (7). Several studies analyze the role of Tamoxifen (an estrogen antagonist) on OSCC lines in relation to the presence/absence of ER (8). In this increasing and suggestive scenario, the purpose of the present study was to evaluate the expression of sexual hormones receptors mRNAs, in particular Estrogen Receptor alpha (ERα) and Androgen Receptor (AR) mRNA in OSCC tissues.

MATERIALS AND METHODS

Tissues

Twenty patients affected by OSCC, not previously treated with chemotherapy and/or irradiation, were consecutively selected for this study. All patients were otherwise healthy; 14 were male and 6 female, with a mean age of 58.2 years (range 38-74). For each patient, two samples of surgical specimen were taken: neoplastic and normal tissue (harvested around the margins of the specimen at least 1 cm from the lesion margins). In all cases, the classification of the tissue was made at the time of surgery by the surgeon (C.G.). Histopathologic examinations were carried out routinely on the samples and the margins were found to be tumor-free. Finally, the samples were quickly frozen by immersion in liquid nitrogen and were stored at –80°C until RNA extraction.

This protocol was approved by an Institutional Review Board and patients gave informed consent.

RNA extraction

Total RNA from OSCC and healthy tissues was extracted with Trizol reagent (Invitrogen, Paisley, UK), a mono-phasic solution of phenol and guanidine isothiocyanate, an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (9). Tissues were homogenized in 1 ml of Trizol reagent; after addition of chloroform for the extraction and precipitation with isopropyl alcohol, the resulting RNA pellet was washed with 70% ethanol and dissolved in sterile water.

Oligonucleotide primers

Specific oligonucleotide primers were designed on mRNA sequences, from NCBI gene bank database, of human androgen receptor (AR, accession No: NM_000044), human estrogen receptor alpha (ERα accession No: NM_000125) and human glyceraldeide-3-phosphate dehydrogenase (GAPDH, accession NM_002046) to amplify respectively 534, 520 and 462 bp of the corresponding coding region (Table I).

RT-PCR, cloning and sequencing

First strand cDNA was synthesized using 3 µg total RNA, 40 ng random exameric primer (Promega Corp., Madison, USA) and 200 U Superscript III (Invitrogen, Paisley, UK) in a total volume of 20 µl according to the manufacturer's instructions (Invitrogen, Paisley, UK). Then, 3 µl of this cDNA template were used for the PCR (final volume 25 µl) with 1.5 mM MgCl2, 1x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl), 0.2 mM dNTP, 2.0 U Taq DNA polymerase (Promega Corp., Madison, USA) and 5 pmol of specific oligonucleotide primers for ERα and AR (see Table I). The same cDNA template (1.5 µl) was also used, as control, to amplify an appropriate region of GAPDH cDNA with specific oligonucleotide primers (Table I). The amplification products for ERα (40 cycles: 94°C for 30", 60°C for 30", 72°C for 45"), for AR (40 cycles: 94°C for 30", 59°C for 30", 72°C for 45") and for GAPDH (30 cycles: 94°C for 30", 60°C for 30", 72°C for 45") were electrophoresed on 1.2% agarose gel in 1xTAE buffer and the quantization was performed by densitometry analysis using GELDOC1.00-UV System (BIORAD, Hercules, CA). The amplification products were purified by a QIAGEN gel extraction kit (QIAGEN, Hilden, Germany) and were cloned into the pGEM-T Easy vector according to the manufacturer's instructions. The inserts were sequenced and the nucleotide sequences were compared to the human sequences in the NCBI gene bank database to verify the specificity of the amplified products.

Statistics

The data were expressed as means ± SD. The Gaussian distribution of continuous variables was assessed by the Shapiro-Wilk normality test and the statistical analysis was performed by the Student's t-test. Probability levels <0.05 were considered statistically significant.

RESULTS

The expression levels of sexual hormone receptors transcripts were studied in OSCC specimens and samples of normal tissue adjacent to the lesion. RT-PCR analysis, carried out on total RNA with the specific oligonucleotide primers (Table I), has revealed the presence of the expected cDNA fragments for ERα (520 bp, Fig.1A-upper panel) and AR (534 bp, Fig.2A-upper panel); in control PCR, using specific primers for GAPDH, the expected 462 bp band was also obtained (Fig.1A and 2A-lower panels). The ERα and AR fragments were eluted from gel, cloned and sequenced to confirm the specificity of the PCR products.

In Fig.1B and 2B we report the averages of the relative densitometric amounts of ERα or AR cDNAs, respectively, versus GAPDH cDNA, obtained on ten specimens and performed in duplicate. This analysis demonstrated that, in OSCC specimens, expressions of ERα and AR transcripts are respectively higher (Fig.1B) and lower (Fig.2B) than in normal tissues.

Table I. *Primer sequences used for PCR analysis*

Primer	Sense	Antisense
Erα (520 bp)	5'-GATGATGGGCTTACTGACCAAC-3'	5'GATGTGGGAGAGGATGAGGAG-3'
AR (534 bp)	5'-ATTGAGCCAGGAGTGGTGTGT-3'	5'-CTTGAGCAGGATGTGGGATT-3'
GAPDH (462 bp)	5'-GTGGATATTGTTGCCATCAATGA-3'	5'-GATGGCATGGACTGTGGTCA-3'

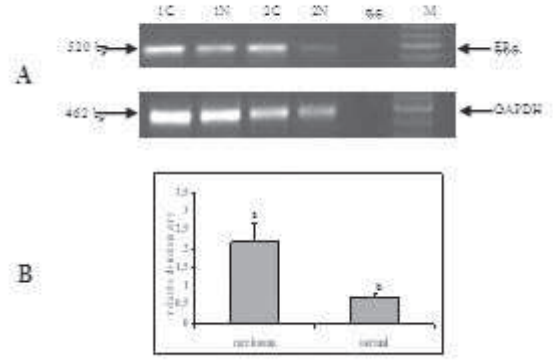

Fig. 1. ***(A)*** *Electrophoretic analysis of ERα (upper panel) and GAPDH (lower panel) transcripts amplified by RT-PCR. The results shown are relative to two specimens. C=carcinoma, N=normal, nc=negative control.* ***(B)*** *Pattern of relative densitometric amounts shown as Era : GAPDH ratios. (a vs. b=p<0.05)*

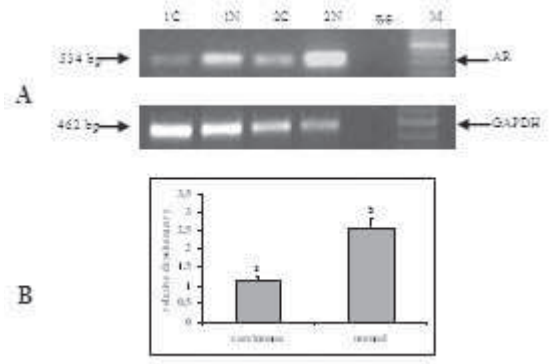

Fig. 2. ***(A)*** *Electrophoretic analysis of AR (upper panel) and GAPDH (lower panel) transcripts amplified by RT-PCR. The results shown are relative to two specimens. C=carcinoma, N=normal, nc=negative control.* ***(B)*** *Pattern of relative densitometric amounts shown as AR : GAPDH ratios. (a vs. b=p<0.05)*

DISCUSSION

The common treatment modalities of OSCC are surgery and/or radiotherapy-chemotherapy. However, the survival rate of the patients has not been improved by the management with these current therapies. (3). The expressions of sexual hormone receptors in some tumors suggest a role for these molecules in their pathogenesis and furnish a possibility of a non-invasive and complementary treatment. It has widely been demonstrated that breast and prostate cancers often express hormone receptors and can be controlled by hormone manipulation (10).

With regard to Head and Neck (HN) district, previous studies evaluated the presence of steroid hormone receptors in HNSCC, mainly with immunohistochemical or radioimmunological analysis, reporting controversial results. A majority of these investigations, conducted principally in relation to laryngeal carcinoma (being the larynx a target organ for androgenic steroids), suggest the hormonal therapy as useful adjunctive strategy of treatment (6).

Here we investigate the presence and the expression levels of sexual steroid receptors, in particular ERα and AR transcripts, in ten OSCC using RT-PCR analysis. Our data indicate that ERα mRNA is more expressed in the OSCC than in control tissues while opposite results have been obtained for AR, in fact, it is less expressed in the OSCC than in the control tissues.

A recent study reported a cross-talking between estrogen receptor and EGFR and suggested that both the estrogen receptor and EGFR pathways together contribute to HNSCC and provided a rationale for potentially targeting these pathways in combination as a new treatment strategy for head and neck cancer (11). Our data also suggest that a OSCC (presence of ERα) may respond to hormonal stimulation.

On other hand our results do not support the hypothesis of a inhibitory effect of estrogen in the growth of cancer cells, as suggested for the esophageal cancer in order to explain the mystery of male predominance (12).

Actually, there are no scientific evidences for the AR role in oral carcinogenesis. In 1994 Nehse and Tunn measured Androgen (AR) and progesterone (PgR) receptors expression in 18 samples of normal oral mucosa and squamous cell carcinoma of the floor of the mouth or the tongue (13). There was a significant difference from

the androgen receptor level in the normal mucosa (13).

Recently Rosa et al. demonstrated that [CAG]n repeat <20 is associated with a more aggressive head and neck cancer subset, in particular in male patients with first-degree family history and oral cavity or oropharyngeal cancers (14) and we know that AR expression is reduced in cells with longer AR CAG repeat alleles (15). The explanation of this correlation is probably that the length of the AR polyglutamine stretch could modify the AR transactivation activity by affecting the associations of the AR with its co-activators in different cell types (16).

In conclusion present data indicate that sex hormones may influence the normal-dysplasia-cancer evolution of the oral mucosa, and could contribute to understanding the molecular pathways of chemotherapy for OSCC through the use of specific antagonists (i.e. SERM).

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