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Evidence for APOBEC3B mRNA and protein expression in oral squamous cell carcinomas

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ABSTRACT

It has been demonstrated that APOBEC3B possesses cytidine deaminase activity, which is likely to result in C-to-T signature mutations. Increased expression of the *APOBEC3B* gene has been shown to correlate with higher incidence of such mutations in various cancer types, such as breast, bladder, lung, and head and neck carcinomas. In the current study, we used in silico methods, immunohistochemistry and qRT-PCR to detect the presence of APOBEC3B signature mutations and examine the levels and patterns of APOBEC3B expression in oral squamous cell carcinomas (OSCCs). Using the Cancer Genome Atlas (TCGA) database, we have found a high incidence of C-to-T transitions in head and neck squamous cell carcinomas (HNSCCs), of which OSCCs constitute the largest subgroup. Additionally, we compared APOBEC3B expression, at both mRNA and protein level, between OSCCs and non-cancerous samples. APOBEC3B was detected in both groups, but nuclear localization was consistent only in normal oral cells. *APOBEC3B* mRNA levels were clearly higher in OSCCs than in controls. These results suggest that while in normal oral cells APOBEC3B has an important nuclear function to fulfill, this activity may be hindered in a subgroup of tumor cells, due to the more prominent localization of the enzyme in the cytoplasm.

1. Introduction

Oral cancer is one of the most common cancers worldwide (Jemal et al., 2011) with oral squamous cell carcinomas (OSCC) representing >90% of all oral malignancies. Despite advances in diagnostic techniques and improvements in treatment modalities, the 5-year survival rate remains relatively low (approximately 50%), mainly because this type of cancer is usually diagnosed at an advanced stage (Warnakulasuriya, 2009).

Tumors derive from the stepwise accumulation of structural and/or functional alterations, such as point mutations, copy number variations, epigenetic changes, deregulation of gene expression, etc. (Vogelstein and Kinzler, 2004). Recently, changes in enzymatic activities have been shown to significantly contribute to DNA damage, resulting in increased mutagenesis (Okazaki et al., 2003; Pasqualucci et al., 2008). The apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3 (APOBEC3) family of enzymes is a major component of innate immunity, bearing the ability to convert cytosines into uracils through deamination (Conticello, 2008). The human APOBEC3 family consists of seven members: APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H (Jarmuz et al., 2002). These cytidine deaminases are capable of defending cells against a variety of viruses and genomic DNA-based parasitic elements (Bogerd et al., 2006; Chiu and Greene, 2008). The best-characterized member, APOBEC3G, accumulates in the cytoplasm and exhibits strong antiviral activity (Harris et al., 2003; Jern et al., 2009; Mbisa et al., 2007; Stenglein et al., 2008). On the contrary, APOBEC3B seems to be predominantly localized in the nucleus, potently inhibiting long interspersed element-1 (LINE-1) and Alu retrotransposition (Bogerd et al., 2006; Stenglein and Harris, 2006). We note here that APOBEC3B is too large to diffuse into the nucleus, hence it is a nucleocytoplasmic shuttle protein, containing a nuclear localization signal (NLS), and a nuclear export signal (NES), which facilitate its transfer from the cytoplasm to the nucleus and vice versa (Bogerd et al., 2006).

Abbreviations: APOBEC, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3; OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; SNP, single nucleotide polymorphism; FFPE, formalin-fixed, paraffin-embedded.

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Since the APOBEC3 cytidine deaminases can act as DNA mutators, concerns have been raised regarding their potential to induce changes in the host human genome (Conticello, 2008), the prime suspect being the seemingly nucleus-based APOBEC3B enzyme. Interestingly, the APOBEC3B protein has been found to favor deamination of cytosine residues flanked by a 5′ thymine residue, resulting in a unique 5′-T**C** mutation signature (Burns et al., 2013a; Shinohara et al., 2012). Recent studies revealed a possible involvement of APOBEC3B in cancer development, due to significantly increasing genomic instability (Burns et al., 2013a; Leonard et al., 2013; Shinohara et al., 2012; Taylor et al., 2013). Further studies demonstrated that APOBEC3B is overexpressed in various types of cancer, including bladder, breast, head and neck, cervical, and lung (adenocarcinoma and squamous cell carcinomas), whereas the upregulation of APOBEC3B correlated with an increased presence of its mutation signature (Burns et al., 2013b; Roberts et al., 2013).

It is not clear, however, whether APOBEC3B localization and potential for mutagenesis also play a role in other cancer types. Thus, we decided to investigate the levels of APOBEC3B expression in OSCC samples, with respect to both mRNA and protein levels. Using in silico approaches, we also sought to identify any possible trends in the emergence of mutated cytosines within the 5′-T**C**W motif, where W corresponds to either A or T.

2. Material and methods

2.1. Material

This is a retrospective analysis of 33 incision biopsies of primary OSCCs, fixed in 10% buffered formalin and embedded in paraffin (FFPE). Data regarding age and sex of the patients, as well as the lesion sites, were obtained from patients' records. Sixteen patients were male (age range 27–78 years, median 53 years) and seventeen female (age range 26–83 years, median 70 years). Lesions were located on the tongue (n = 27), gingivae (n = 2), palate (n = 2), mouth floor (n = 1), and buccal mucosa (n = 1). Data regarding smoking and alcohol consumption were not available. Tumors were graded according to the World Health Organization (WHO) classification into well (n = 13), moderately (n = 14), and poorly (n = 6) differentiated. Due to the origin of our material (incision biopsies), stage and survival data were not available. Thirteen cases of similarly processed non-cancerous irritation fibromas (n = 13) were utilized as normal controls (Table 1).

The study was conducted in accordance with the Declaration of Helsinki principles and was approved by the Ethics and Research Committee of the Dental School of the University of Athens, Greece.

2.2. Single-point mutation patterns in head and neck squamous cell carcinomas

We collected somatic mutation data from The Cancer Genome Atlas (TCGA). The data is currently hosted in the National Cancer Institute data portal. The portal provides a platform for researchers to search, download, and analyze data sets related to various cancer types. Each set contains clinical information, genomic characterization data, and high level sequence analysis of tumor genomes. We downloaded data for 510 samples (https://gdc-portal.nci.nih.gov/files/64683606-b957-4478-a7d5-673de68b0341) pertaining to head and neck squamous cell carcinomas (HNSCC). More particularly, we used the available MAF file (Mutation Annotation Format), which lists mutations (mostly SNPs) along with various relevant data (such as mutation position, mutation type, chromosome and strand) pertaining to them. From the MAF file, we collected all SNPs (Single-Nucleotide Polymorphisms) and compared them with the GRCh38 human data set, available from the UCSC Human Genome Browser (Kent et al., 2002). We additionally collected flanking 3′ and 5′ nucleotides for each SNP. We searched the resulting list of trinucleotide mutations where the middle nucleotide is substituted, for the APOBEC3B-specific signature T**C**W > T**T**W (where W can be either Adenine or Thymine) in order to locate APOBEC3B specific mutations. It should be noted that the W**G**A > W**A**A mutations, representing the reverse complement T**C**W > T**T**W on the opposite DNA strand, were also included.

Table 1
Clinical data.

Number of patients	46
Gender	
Male	16
Median age at diagnosis	53
Female	17
Median age at diagnosis	70
OSCC	33
Well differentiated	13
Moderately differentiated	14
Poorly differentiated	6
Irritation fibromas	13
Median age at diagnosis	56

2.3. Immunohistochemistry

Immunohistochemical analysis was performed on 4–5 μm-thick FFPE tissue sections that were treated overnight with a polyclonal antibody (# orb155694, Biorbyt, Cambridge, UK) raised specifically against residues 1–100 of the human APOBEC3B protein, at a dilution of 1:150. After testing several antigen retrieval protocols, we determined that optimal results were obtained when using a Tris-EDTA pH 9.0 solution, in combination with the PT Link pre-treatment module (DAKO, Agilent Technologies, CA, USA) for deparaffinization, rehydration and epitope retrieval. EnVision (DAKO), a standard two-step visualization system, was used. Omission of the primary antibody served as the negative control and breast carcinoma FFPE samples were used as positive controls.

2.4. Evaluation of immunohistochemistry

Two independent investigators, from whom the origin of the FFPE lesions was withheld, evaluated the extent and intensity of immunoreactivity according to a three-scale semi-quantitative system:

- Extent: we used the categorization system described elsewhere (Vered et al., 2006). Samples where <5% cells had been stained were classified as “grade 0”, samples where the percentage was between 5 and 50% were classified as “grade 1”, and samples with percentages higher than 50% were classified as “grade 2”.
- Intensity: 0 for absence of color, 1 for yellow color and 2 for dark color.
- The total immunohistochemistry score (IS) was calculated using the formula: IS = *extent* × *intensity*, and characterized as absent (IS = 0), weak-moderate (IS = 1 or 2), and intense (IS = 4).

There were no discordant results between the investigators.

2.5. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from formalin-fixed, paraffin-embedded samples of OSCC and traumatic fibromas, using the PureLink FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). 1 μg of total RNA was reverse transcribed by SuperScript II Reverse Transcriptase using random primers and RNase Out Ribonuclease Inhibitor (Invitrogen). The Kapa SYBR Fast qPCR Kit (Kapa Biosystems, Cape Town, South Africa) was used for real-time PCR in an Mx3000P qPCR system (Agilent Technologies). Primers used