Oral cancer, cigarette smoke and mitochondrial 18 kDa translocator protein (TSPO) – In vitro, in vivo, salivary analysis

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A B S T R A C T

Oral cancer features high rates of mortality and morbidity, and is in dire need for new approaches. In the present study we analyzed 18 kDa translocator protein (TSPO) expression in oral (tongue) cancer tumors by immunohistochemistry. We also assayed TSPO binding in human tongue cancer cell lines and in the cellular fraction of saliva from tongue cancer patients, heavy cigarette smokers, and non-smoking healthy people as controls. Concurrently, TSPO protein levels, cell viability, mitochondrial membrane potential (Δψm), and general protein levels were analyzed. TSPO expression could be significantly enhanced in oral cancer tumors, compared to unaffected adjacent tissue. We also found that five-year survival probability dropped from 65% in patients with TSPO negative tumors to 7% in patients with highly expressed TSPO (p<0.001). TSPO binding capacity was also pronounced in the human oral cancer cell lines SCC-25 and SCC-15 (3133±461 pmol/mg protein and 6956±540 pmol/mg protein, respectively). Binding decreased by 56% and 72%, in the SCC-25 and SCC-15 cell lines, respectively (p<0.05) following CS exposure in cell culture. In the cellular fraction of saliva of heavy smokers TSPO binding was lower than in non-smokers (by 30%, p<0.001). Also the cellular fraction of saliva exposed to CS in vitro showed decreased TSPO binding compared to unexposed saliva (by 30%, p<0.001). Interestingly, oral cancer patients also displayed significantly lower TSPO binding in the cellular fraction of saliva compared to healthy controls (by 40%, p<0.01). Our results suggest that low TSPO binding found in the cellular fraction of saliva may depend on genetic background as well as result from exposure to CS. We suggest that this may be related to a predisposition for occurrence of oral cancer.

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1. Introduction

Oral cancer, which is induced primarily by cigarette smoke (CS), is the sixth most common malignancy. The carcinogenic effect of cigarette smoke is enhanced by salivary modulation [1]. Oral cancer is characterized by a poor 5-year survival rate of only 50% and a high rate of morbidity, which have not changed significantly in the past half-century [2–4]; thus, a better understanding of the biological nature of this aggressive disease is mandatory. Accordingly, we examined a potential novel player in the field of oral cancer, the translocator protein (18 kDa) (TSPO). The TSPO, which was previously called peripheral-type benzodiazepine receptor (PBR), was first identified in 1977 as a diazepam binding site outside the brain [5]. This led to its assignment as ‘peripheral-type’ benzodiazepine receptor (PBR), to distinguish it from the central benzodiazepine receptor (CBR). While the CBR is primarily located on cell membranes of neurons in the CNS, the TSPO’s location is mostly on mitochondrial membranes in various cell types. The TSPO has been detected in various densities in the majority of tissues tested [6,7]. TSPO is located mainly in the outer mitochondrial membrane and is closely associated with the mitochondrial permeability transition pore (MPTP). Increased binding levels of TSPO have been observed in various human cancers [6,8–13]. The expression and binding levels of TSPO have been found to correlate with the tumorigenicity of various cancer cell lines [14]. Weisinger et al. [15] induced TSPO underexpression by using the stable anti-sense knockout approach in the MA-10 Leydig cell line. The resultant clones demonstrated increased tumorigenicity, both in vitro (loss of contact inhibition and growth in soft agar) and in vivo (increased mortality after back grafting into isogenic mice) assays. Other studies applying TSPO knockdown by genetic
manipulation by various methods to various types of cancer cells, as well as application of TSPO specific ligands, showed that the TSPO plays an important role in the induction of all the aspects of the mitochondrial apoptosis pathway, as well as necrosis [16–20].

In the current study we used 3 models to study the potential pathogenic role of TSPO in oral cancer. These models included oral cancer tissues, oral cancer cell lines, and the cellular fraction of saliva of oral cancer patients. The salivary analysis was also conducted in non-smoking healthy individuals as well as in heavy smokers, both in vivo and in vitro.

2. Patients and methods

2.1. Experimental design

The in vivo part of the study was based on an analysis of 69 patients (33 males and 36 females aged 65.7 ± 15.2 years) with tongue cancer whose archival paraffin-embedded pathological material was available for immunohistochemical staining analysis of TSPO. In 41 cases we studied the TSPO staining levels together with those of Skp2 and p27 proteins. The analysis was performed as previously described [21]. Seventeen patients were diagnosed with stage 1 cancer. The analysis was performed as previously described [1]. Previous studies have shown, by measuring the NO2-concentrations in the media, that the time of exposure to the smoke correlates directly with the level of exposure to cigarette smoke [31–33]. Using the same system we also exposed oral cancer cell lines to cigarette smoke. Using these various models allowing studying oral cancer both at the tissue level (specimens of tumors) and at the cellular level (cells in saliva and oral cancer cell lines in cell culture medium).

2.2. Immunohistochemical analysis and Western blot

The preparation and specificity of the antiserum against TSPO were described previously [17,18,20]. This antiserum was used for the immunohistochemistry and Western blot analysis of the present study. Western blot analysis with this antiserum was performed as described in detail previously [17,18,20]. Labeling of β-actin was used as a loading control. Labeling for the 32 kDa voltage dependent anion channel (VDAC) was done as routinely performed in our laboratory (e.g. [17,20,27]) as it is a protein closely associated with the TSPO.

For immunohistochemical staining of oral cancer specimens and control tissue, five micron sections were deparaffinized with xylene and rehydrated in a series of ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 20 min. For epitope retrieval, slides were heated in a microwave oven at 92 °C for 20 min in a Tris-EDTA-buffer pH 8.0. After cooling, slides were washed in distilled water and then in phosphate-buffered saline (pH 7.4). Slides were incubated overnight at 4 °C with the primary antiserum directed against TSPO diluted 1:100. Staining was completed with a Histostain-Plus kit (Zymed laboratories, CA). Color reaction product was developed with aminoethylcarbazole as the chromogen. All sections were counterstained with hematoxylin, dehydrated, and cover slipped. Incubations with phosphate-buffered saline containing 1% bovine serum albumin instead of the primary antibody were used as negative controls. Skp2 and p27 antisera were applied with similar immunostaining procedures, as described previously [21,34].

Staining intensity was examined microscopically, double blind, as neither the person examining the sections nor the person providing the sections to the examiner knew the patient origin of the samples. Weak cytoplasmic staining intensity for TSPO of tumor cells was graded < 1 (weak). Weak staining meant that less than 50% of tumor cells were stained. Moderate to strong cytoplasmic staining of tumor cells was graded > 1 (moderate-strong). In all cases of moderate to strong staining for TSPO more than 50% of tumor cells were stained. Skp2 and p27 staining intensity was determined as described previously i.e. at least 500 tumor cell nuclei were counted and from this population the percentage of Skp2 and p27 positive cells was calculated [21,34].

2.3. Cell viability

Cell viability was determined in saliva samples using the Trypan Blue exclusion test. Following treatment, saliva samples were centrifuged at 800 × g for 10 min and the pellet containing the cellular fraction was suspended in phosphate-buffered saline (PBS). Cell counting was performed using an inverted microscope and a hemocytometer. Both viable and non-viable cells were counted and the percentage of viable cells was calculated from the total number of cells.

2.4. Mitochondrial transmembrane potential analysis

The specific stain JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide) was used to assay changes in the ΔΨm following exposure oral cancer cell lines to cigarette smoke, as described in detail previously [17,20,35]. Briefly, samples of confluent cells were collected and centrifuged at 1200 × g for 10 min. Cell pellets were re-suspended in 1 μg/ml JC-1 solution in PBS and incubated at
37 °C for 30 min in the dark. After incubation, the cells were centrifuged at 1200 × g for 10 min, and re-suspended in 0.5 ml PBS. Then, the cell suspensions were transferred into 5 ml FALCON® FACS tubes and analyzed with the flow cytometer using CellQuest software.

2.5. TSPO binding analysis of saliva and oral cancer cells

Assays of [3H] PK 11195 binding to membranes of the SCC-15 and SCC-25 cells for Scatchard analysis were conducted, according to methods described previously [14,16,25]. The reaction mixture contained 400 µl of the homogenized samples and 25 µl of [3H]PK 11195 (final concentration of 0.2–6 nM), in the absence (total binding) or in the presence (non-specific binding) of 10 µM unlabeled PK 11195. After 80 min incubation at 4 °C, the samples were filtered through Whatman CF/C filters using a vacuum system, washed three times with 4 ml of 5 mM ice cold phosphate buffer and placed in vials containing 4 ml of CytoScint™ (MP Biomedicals, Costa Mesa, CA). Radioactivity was counted after 12 h with a liquid scintillation analyzer. Specific binding was obtained by subtracting non-specific binding from total binding. The maximal binding capacity (Bmax) and equilibrium dissociation constant (Kd), were calculated from the saturation curve of [3H]PK 11195 binding, using Scatchard analysis.

For binding analysis of the cellular fraction of saliva, saliva samples were thawed and immediately centrifuged (800 × g, 10 min, 4 °C). The pellet, containing cells present in the saliva, was suspended in ice cold PBS, and then homogenized using a Kinematika Polytron (Luzerne, Switzerland) (setting 6) for 10 s. Total protein amount was determined, according to Bradford et al. [24]. TSPO binding assays of the saliva were conducted at concentrations of 6 nM and 12 nM of [3H] PK 11195.

2.6. Statistical analysis

For categorical variables, frequencies and percentages were calculated. Distributions for categorical variables were compared and analyzed by the Fisher–Irwin exact test (small sample). For continuous variables, ranges, means, standard deviation, and standard error were calculated. The results of continuous variables between subgroups of patients were compared and analyzed by one way analysis of variance. The “Kaplan Meier estimate” was used to calculate the probability of survival rates as a function of time. The “Log Rank test” was used to compare between survival curves. p < 0.05 was taken as indicating statistically significant differences.

3. Results

3.1. TSPO levels in tongue cancer samples and clinical characteristics

Of the 69 oral cancer patients analyzed, oral tumor tissue of 12 patients (18%) stained negatively (=0) for TSPO while the other 57 patients (82%) the oral tumor tissue stained positively. The TSPO positive group was further categorized according to the intensity of staining: weak staining (<1) was found in 52% (36/69) of the specimens and moderate to strong staining (>1) in 30% (21/69). The TSPO staining was cytoplasmatic and slightly granular in the tumor tissue (Fig. 1), while the adjacent, normal-looking tissue was not stained by the anti-TSPO antibody (not shown), thus serving as internal controls. TSPO immunostaining was not significantly different between males and females nor was it significantly affected by age. Neither did the TSPO level significantly correlate with the grading (Fig. 1) nor with the T, N or M values.

3.2. Prognostic value of TSPO for oral cancer

In order to determine the prognostic value of TSPO for tongue cancer patients, we analyzed their cumulative survival according to TSPO expression levels. The intensity of TSPO staining correlated strongly with patient mortality. While 65% of the patients negative for TSPO and 57% of the patients with low (<1) TSPO levels in the tongue tissue were still alive at 60 months, only 7% of the patients with moderate to strong (>1) TSPO labeling of the tongue tumor tissue survived the 60 month period (p<0.001) (Fig. 2).

3.3. Correlations between TSPO, Skp2 and p27 expression levels in oral cancer tissue

Oral tumor TSPO staining levels correlated negatively with Skp2 levels and positively with p27. In particular, the mean Skp2 staining level of the TSPO positive (>0) specimens was significantly lower than that of the TSPO negative specimens (=0), 14.5 ± 2.1% (in 32 patients) vs. 21.5 ± 4.7% (in 7 patients), respectively (p = 0.05) (Table 1A). In contrast, the mean p27 staining level of the TSPO positive specimens was significantly higher than that of the TSPO negative specimens, 39 ± 2.3% (in 34 patients) vs. 27 ± 4.2% (in 7 patients), respectively (p < 0.05) (Table 1B).

3.4. TSPO in the SCC-25 and SCC-15 cells

We applied Western blot to determine whether TSPO is present in SCC-25 and SCC-15 cells. TSPO indeed are expressed in these human oral cancer cells. A representative Western blot of SCC-25 cells is presented in Fig. 3.
3.5. TSPO binding characteristics of [3H]PK 11195 in the SCC-25 and SCC-15 oral cancer cell lines

Binding assays with [3H]PK 11195 showed $B_{\text{max}}$ and $K_d$ values for the SCC-25 cells of 3133 ± 643 fmol/mg protein and 5.7 ± 2.0 nM, respectively (mean ± SD, $n$ = 7). The $B_{\text{max}}$ and $K_d$ values of the SCC-15 cells were 6956 ± 549 fmol/mg protein and 5.9 ± 4.6 nM, respectively (mean ± SD, $n$ = 16). Non-specific binding of [3H]PK 11195 to human oral cancer cells was one third of the total binding. Representative Scatchard analysis of saturation curves of [3H]PK 11195 specific binding to TSPO in human oral cancer cell lines SCC-25 and SCC-15 is presented in Fig. 4.

3.6. TSPO binding and protein concentration following exposure of SCC-25 and SCC-15 cells to cigarette smoke

With binding assays of SCC-25 cells that were exposed to CS for 90 min, using [3H]PK 11195 as a radioligand (final concentrations 3 nM and 6 nM) we found a significant decrease of 72% in TSPO binding at the concentration of 3 nM [3H]PK 11195 in the CS-exposed cells, as compared to controls ($n$ = 6; $p$ = 0.01; data not shown). Similarly, the mean TSPO binding at a final concentration of 6 nM was lower by 56% in the CS-exposed cells ($n$ = 6; $p$ = 0.03; data not shown). Using a concentration of 3 nM [3H]PK 11195 in CS-exposed SCC-15 cells, we also found a significant decrease of 64% in TSPO binding as compared to controls (data not shown). Total protein concentrations in SCC-25 and SCC-15 cells exposed to cigarette smoke (CS) for 90 min did not differ from controls, as measured by the method of Bradford et al. [24].

Table 1

<table>
<thead>
<tr>
<th>TSPO level</th>
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<tr>
<td>A. TSPO and Skp2 staining levels in tongue cancer specimens</td>
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</tr>
<tr>
<td>Number of oral cancer patients</td>
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<td>32</td>
</tr>
<tr>
<td>Mean % of Skp2 labeled cells</td>
<td>21.5</td>
<td>14.5*</td>
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<tr>
<td>SE</td>
<td>4.77</td>
<td>2.10</td>
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<tr>
<td>*$p$ = 0.05</td>
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<tr>
<td>B. TSPO and p27 staining levels in tongue cancer specimens</td>
<td></td>
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<tr>
<td>TSPO level</td>
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<td>&gt;0</td>
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<tr>
<td>Number of oral cancer patients</td>
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<td>34</td>
</tr>
<tr>
<td>Mean % of p27 labeled cells</td>
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<td>39.0*</td>
</tr>
<tr>
<td>SE</td>
<td>4.2</td>
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<td>*$p$ &lt; 0.05</td>
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The percentage of Skp2 labeled cells is significantly lower in tongue cancer specimens with enhanced TSPO levels (A), while the percentage of p27 levels is significantly higher in tongue cancer specimens with enhanced TSPO levels (B).

3.7. Collapse of the mitochondrial transmembrane potential in SCC-25 cells exposed to cigarette smoke

The mitochondrial potential stability assays as assayed in SCC-25 cells showed that cigarette smoke caused collapse of the mitochondrial membrane potential ($\Delta \psi_m$) in 80% of the cells, which present a significant increase from to control (Fig. 5). Collapse of the $\Delta \psi_m$ can lead to cell death, including apoptosis [17,20].

Fig. 2. Correlation between TSPO levels in oral cancer tissue and survival rates. Cumulative survival rate and TSPO expression ($'0'$ vs. $'1'$ vs. $'>1'$) ($n$ = 69). The probability of survival of patients with TSPO level ($=0$) at 60 months was 65% while the probability of survival of patients with TSPO level ($>1$) at 60 months was 7% only. ***$p$ < 0.001 for $>1$ vs. $=0$.

Fig. 3. Protein expression of TSPO, VDAC, and beta-actin in oral cancer cell lines. A representative Western blot analysis of TSPO, VDAC and beta-actin proteins in the SCC-25 cell line growing under normal physiological conditions.

Fig. 4. TSPO binding characteristics in oral cancer cell lines. Scatchard plots (A,B) of [3H]PK 11195 (0.2–6 nM final concentration) binding to SCC-25 human oral cancer cell line (panels A and B respectively) and to SCC-15 human oral cancer cell line, ($n$ = 16). Explanation of abbreviations: B = concentration of Bound ligand, B/F = concentration of Bound ligand over concentration of Free ligand.
3.8. TSPO binding, cell viability, and total protein in the cellular fraction of saliva of heavy smokers

Similar to the effects of CS on the TSPO binding in the oral cancer cell lines, the cellular fraction of saliva from heavy smokers showed that the mean±SE TSPO binding with the [3H]PK 11195 ligand (at a final concentration of 6 nM) was reduced significantly by 53% (9 smokers), as compared to the control group (16 non-smokers) (p<0.05) (Fig. 6A). Western blot analyses of TSPO, VDAC and β-actin proteins in the cellular fraction of saliva from non-smoking individuals and smoking individuals, revealed a lower expression of the TSPO and VDAC proteins in heavy smokers (Fig. 6B). The mean±SE percentage values of viable salivary cells were found to be similar in both groups, with 36±4% in control saliva (16 non-smokers) and 37±4% in smokers saliva (6 smokers). Also the mean±SE of total protein concentration in saliva of non-smoking controls (16 non-smokers) and heavy smokers (9 smokers) were similar, i.e. 0.31±0.08 mg/ml and 0.44±0.16 mg/ml respectively.

3.9. In vitro analysis of TSPO binding, cell viability, and total protein levels in saliva exposed to CS

Saliva samples which had been collected from healthy non-smoking volunteers were exposed to CS in vitro and then the cellular fraction subjected to TSPO binding analysis. The binding analysis with a single [3H]PK 11195 (6 nM final concentration), in control and CS-exposed saliva revealed a significant 30% reduction of the TSPO binding (n=34, p<0.001) in response to CS exposure, compared to sham controls (Fig. 7). Cigarette smoke did not appear to affect cell viability in saliva. The mean percentage values of viable cells were found to be similar in both groups, with 32.0±3.8% in control saliva (n=25) and 36.0±3.5% (n=25) in cigarette smoke-exposed saliva. The mean total protein concentrations in the cellular fractions from the control and the cigarette smoke-exposed samples were similar (data not shown).

3.10. TSPO binding, cell viability, and total protein levels in saliva of oral cancer patients

We conducted two separate binding experiments on saliva samples of oral cancer patients and healthy controls, using [3H]PK 11195 as a radioligand. In the first experiment we used two concentrations of [3H]PK 11195 (6 nM and 12 nM), and in the second experiment we used 6 nM only. In the first experiment (Fig. 8) we compared 13 salivary samples of oral cancer patients with 6 salivary samples of controls, and found a significant 38% decrease in TSPO binding at the concentration of 6 nM [3H]PK 11195 (p<0.05) in the cellular fraction of saliva from oral cancer patients, as compared to controls. Similarly, the mean TSPO binding at a final concentration of 12 nM was significantly lower by 41% in the cancer patients (p<0.05) (Fig. 8). In the second experiment, using 6 nM final concentration of [3H]PK 11195, the decrease in TSPO binding in the cellular fraction from saliva of oral cancer patients was 40% (n=12 for healthy controls, n=7 for oral cancer patients, p<0.05).

Viability assays with Trypan blue showed that the mean±SE percentage values of viable cells were similar in both groups, with 48±7% in saliva of controls (n=16) and 40±4% in saliva of oral cancer patients (n=7). Also the mean±SE total protein concentration [24] in the cellular fraction of saliva from healthy controls...
that activation of the TSPO affects the activity of the Fo subunit of the ROS generation at mitochondrial levels [19,20]. Recently, it was found that the mechanism whereby TSPO initiates the mitochondrial apoptosis cascade includes oxidation of cardiolipins i.e. addition, it was found that the mechanism whereby TSPO initiates the TSPO can serve to activate the mitochondrial apoptosis cascade. In TSPO knockdown, and various types of TSPO ligands indicate that the TSPO ligands PK 11195 and Ro5 4864 [14,17,19,20]. Thus, these and prostate cancer [6,8,9,11–14,37]. Experimental studies inducing over-expression of CoCl2 lead to cell death of brain cancer cells, however, low concentrations of CoCl2 led to reductions in TSPO levels, while high concentrations of CoCl2 enhanced TSPO levels [20]. Only application of TSPO knockdown showed that TSPO was required for apoptosis in this paradigm [20]. Furthermore, while the apoptosis can be considered an important feature of carcinogenesis, it is also well known that the TSPO is involved in other important functions, such as cholesterol transport over the outer mitochondrial membrane and modulation of steroidogenesis, inflammation, and immune response [39,45].

Analysis of TSPO in the oral cancer cell lines, SCC-25 and SCC-15, revealed that TSPO is well expressed in these cells. The oral cancer cell lines were sensitive regarding viability when exposed to cigarette smoke, in correlations with reductions in TSPO binding. For example, we found that cigarette smoke causes collapse of the mitochondrial membrane potential in SCC-25 cells. Regarding cells in saliva, exposure of salivary samples to cigarette smoke did not appear to affect viability of cells in these salivary samples, while simultaneously reductions in TSPO binding were observed. Similarly, oral cancer patients showed relatively low levels of TSPO in the cellular fraction of salivary samples, without concomitant effects on cell viability in these salivary samples. It is not clear why viability of cells in saliva is not affected by cigarette smoke, while cultured oral cancer cell lines appear to be very sensitive to cigarette smoke. More detailed studies are required to resolve these questions.
which is the mechanism whereby reduced TSPO levels in the cellular fraction of saliva may potentially contribute to oral cancer tumorigenesis. Furthermore, our data presently do not resolve whether reduced TSPO binding in the cellular fraction of saliva from cigarette smokers is due to an acute effect of cigarette smoke exposure, or a chronic effect due to the chronic cigarette smoke exposure. More studies are needed to answer these questions. Furthermore, the specific molecular biological mechanism by which cigarette smoke reduces TSPO binding, as currently shown in the SCC-25 and SCC-15 cells, and salivary cells, is yet to be elucidated. Previous studies have suggested that oxidative stress can affect the affinity of the TSPO due to the formation of homomeric TSPO multimers [47], this may be part of the mechanism whereby cigarette smoke may affect TSPO binding. A potential role of TSPO in the pathogenesis of oral cancer may originate from its location at chromosome 22q13.1. QRT-PCR has shown that specific region deletion on 22q13 is related to prognosis in oral cancer. Furthermore, the incidence of loss of heterozygosity (LOH) at chromosome 22q is high and is associated with the carcinogenesis of oral cancer [48,49]. Different tumor suppressor genes related also to oral cancer are located in distinct regions on chromosome 22q [50]. Possibly, TSPO is part of this family of tumor suppressor genes.

In summary, our results indicate for the first time that increased TSPO levels in oral cancer tissue may be correlated with oral cancer mortality prognosis. On the other hand, reductions in TSPO binding in the cellular fraction of saliva in the oral cavity may contribute to the occurrence of cancer. This role of salivary TSPO may be based on induced reductions in the TSPO normal binding and/or protein expression rendered by inborn defects or by a later exposure to carcinogens such as those contained in cigarette smoke (Fig. 9). Thus, our studies suggest that TSPO malfunction may contribute to carcinogenesis, implying that the TSPO may be targeted as a venue for treatment of oral cancer. More studies correlating TSPO functions with oral cancer prognosis and diagnosis are needed. In particular, causal relationships need to be established in this area.

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