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"NEW ROUTES FOR RISK STRATIFICATION OF PATIENTS WITH HPV-NEGATIVE ORAL SQUAMOUS CELL CARCINOMA – EXPLORING THE TUMOR-MICROENVIRONMENT INTERFACE"

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1 Introduction

1.1 Head and Neck Cancers

Head and Neck Cancers (HNCs) comprise a wide range of neoplasms originating from the lining epithelium of oral cavity, oropharynx, larynx, nasal cavity, paranasal sinuses, nasopharynx, and salivary glands. The majority of HNCs are represented by squamous cell carcinomas (Head and Neck Squamous Cell Carcinoma, HNSCC) [1].

1.2 Squamous cell carcinoma of oral cavity (OSCC)

1.2.1 Epidemiology

HNSCCs mainly comprise squamous cell carcinomas of the oral cavity (OSCC). In the United States, about 50,000 new cases of oral cancer were estimated in 2016, and about 10.000 deaths were caused by this disease. The worldwide OSCC incidence is approximately 300.000 new cases per year, with an annual death rate of approximately 145.000. The highest incidence rates were found in Melanesia, South-Central Asia and Central and Eastern Europe [2] [3]. About Italy, there have been observed in 2016 10.000 new cases (with 100.000 cases per year), with a prevalence in men [4].

1.2.2 Risk factors

OSCC is mostly associated to tobacco and to alcohol abuse as well as low fiber and vitamins diet and poor oral hygiene [5-9]. A persistent Human Papillomavirus (HPV) infection is a further emerging etiological factor for a subset of SCC restricted to the oropharyngeal region [10].

1.2.3 Biological behavior of OSCC

Whereas HPV-related OSCC are characterized by a significantly slow progression and high response to chemo- and radiotherapy [11], the “classical, tobacco smoking/alcohol related” OSCC are intrinsically highly aggressive and almost always chemo- and radio resistant when in advanced stage. To date, there is still an urgent need for new prognostic and predictive biomarkers, for these deadly cancers, that kill about 50% of patients with metastatic disease.

1.2.4 Classification, grading and staging

The oral cavity extends from the skin–vermilion junction of the lips to the junction of the hard and soft palate above and to the line of circumvallate papillae below and is divided into the following specific sites: mucosal lip, buccal mucosa, lower alveolar ridge, upper alveolar ridge, retromolar trigone, floor of the mouth, hard palate and anterior two-thirds of the tongue (oral tongue) [12]. There are 8 histologic variants of squamous cell carcinoma:

conventional, verrucous, spindle cell, papillary, basaloid squamous, adenosquamous, adenoid, and lymphoepithelial. The degree of differentiation ranges from G1 (well differentiated), to G2 (moderately differentiated) and G3 (poorly differentiated). The pathological staging after surgery adds information regarding the prognosis and is important for the choice of post-operative treatment. The pathological report will encompass informations on the size, number and level of eventually involved lymph nodes, capsular infiltration will also be specified. Postoperative staging must also provide information about the resection margins (infiltration and adequacy), vascular and/or lymphatic embolization and perineural involvement. Only the pathological staging can provide information about the radicality (R0) of the intervention. In stage I and II disease, the main clinical problem is the locoregional disease control. The risk of metastasis to the lymph nodes depends on the location (more or less pronounced lymphatic drainage) and, in some districts, increases with the thickness of the primary tumor. The risk is especially high for tongue and mouth floor cancers [13]. A meta-analysis of the literature (16 studies, 1136 patients) [14] described the relationship between the tumor thickness and locoregional lymph node metastases identifying a cut-off of 4 mm (on the pathological specimen fixed after surgery) to define the risk of lymph node metastases in tumors of the lingual body.

1.3 FK506 binding proteins (FKBPs)

1.3.1. FKBP51

The FK506 binding protein 51 (FKBP-51) is a member of the FK506 binding proteins (FKBPs), a family of immunophilins, target of immunosuppressive drugs such as rapamycin and tacrolimus (FK506) [15]. FKBPs have another hallmark, the peptidyl-prolyl isomerase activity (PPIase) responsible for interconversion from cis to trans form and vice versa of peptide bonds containing proline [15, 16]. To date, 15 different forms of FKBPs have been identified, distinguished according to their molecular weight [17]. FKBP-12, an ubiquitous immunophilin of 11.8 kDa highly conserved in eukaryotes, was the first FKBP described in detail. The FK domain at the amino-terminal region of FKBPs, is responsible for binding with immunosuppressive drug and PPIase. At the carboxy-terminal, reside tetratricopeptide domains (TPR) involved in protein-protein interactions, in particular with the heat shock proteins HSP90 and HSP70 and steroid hormones receptors. The presence of different functional domains is indicative of the ability of these proteins to carry out different essential cellular functions [15-17]. FKBP-51 is involved in several cellular growth and differentiation regulatory mechanisms, this suggesting its key role in both physiological and pathological conditions, as in pre-neoplastic and neoplastic lesions. FKBP-51 has been cloned in 1995 in mice, restricted to T lymphocytes [18]. In humans, several studies confirmed its expression either in T lymphocytes and in several tissues [19]. FKBP-51 is encoded by FKBP5 gene, located on the short arm of chromosome 6 (6p21.31), consisting of 13 exons and 12 introns for more than 150 kb. FKBP5 gene is similar to FKBP4 gene coding for the immunophilin FKBP-52, indicating a same derivation from a common ancestral gene present in invertebrates [20].

FKBP-51 participates in the negative control of the immune system induced by immunosuppressive drugs antagonists of calcineurin (CaN), as FK506 (or tacrolimus). The CaN is a serine / threonine phosphatase, Ca2 +/Calmodulin-dependent, essential for the transduction of TCR signaling in T lymphocytes by activation of the NF-AT transcription factor (Nuclear Factor of Activated T lymphocytes). NF-AT, dephosphorylated by CaN, is active and able to translocate from cytoplasm to nucleus, resulting transcription of the IL-2 gene and other molecules involved in the activation T lymphocytes. FK506 interacts with FKBP-12 or FKBP-51, forming a FK506-immunophilin complex able to inhibit CaN and the subsequent dephosphorylation and activation of NF-aT, blocking T lymphocytes proliferation [18, 21, 22]. FKBP-51 interacts also with intracellular glucocorticoids, estrogen/ progesterone and mineralocorticoid receptors, forming a hetero complex with Hsp70-Hsp90 proteins. FKBP-51 downregulates the response to steroid hormones, except for androgen receptor (AR). Several studies have etiologically related prostate cancer to the overexpression or otherwise increased activity of the immunophilin [23]. In the nervous system, FKBP-51 regulates the clearance of tau protein and stabilizes microtubules contributing to the integrity of the cytoskeleton and survival of neurons. The accumulation of tau protein leads to cytoskeletal alterations typical of a group of neurodegenerative diseases, among which also the Alzheimer's disease [24-26].

Another transduction signaling pathway that involves FKBP-51 is the PI3K / AKT / mTOR signal that has a critical function in cell proliferation, cell cycle progression, apoptosis and metabolism. FKBP-51 down regulates this pathway, favoring the interaction between AKT kinase and its down-regulator PHLPP, catalyzing the dephosphorylation of AKT at the level of S473. The FKBP51 down-regulation reduces the interaction between AKT and PHLPP and increases the levels of phosphorylated (activated) AKT, resulting in increased cell survival and chemo-resistance. The identification of its function as scaffold in the regulation of AKT permitted to hypothesize a role of this immunophilin as a potential biomarker of tumorigenesis and chemoresistance [27]. Recently, an essential role played by FKBP-51 also in the activation of NF-kB and NF-kB-regulated genes has emerged. FKBP-51, through its isomerase activity on the IKKα subunit, allows the correct functioning of the IκB kinase complex (IKK). This function, through phosphorylation and ubiquitin-dependent degradation of the inhibitor IκB, induces the activation of the pathway of NF-kB [28- 30]. FKBP-51 can regulate the activation of NF-kB even through a further indirect type of mechanism, involving the inhibition of the response to steroid hormones. Glucocorticoids are, in fact, potent inhibitors of NF-kB pathway, since they induce the transcription of the inhibitor IκBα that sequesters NF-kB into an inactive cytoplasmic complex [31]. FKBP-51, downregulating the response to glucocorticoids, promotes the activation of NF-kB.

1.3.2 FKBP51 and NF-kB

Several scientific evidences supported the hypothesis of a possible role of FKBP-51 in the control of NF-kB pathway. Giraudier and colleagues in 2002, for the first time disclosed in idiopathic myelofibrosis, a possible

interaction between FKBP-51 and NF-kB. The authors correlated megakaryocytes' growth, independent from cytokines, with the overexpression of FKBP-51, able to inhibit the calcineurin, involved in dephosphorylation of IκB and in the control of NF-kB. FKBP-51, by activating NF-kB, promotes the expression of anti-apoptotic molecules such as Bcl-xL responsible for megakaryocytes survival [32].

Bouwmeester et al co-purified FKBP-51 with IKKα, IKKε, TAK1 and MEKK1 and postulated FKBP51 as a potential cofactor of several kinases involved in the regulation of NF-kB [33]. In 2004, a further study described FKBP-51 as an essential factor in the activation of NF-kB induced by chemotherapeutic agents in melanoma [28]. The authors showed that the immunosuppressant rapamycin was able to counteract the activation of NF-kB induced by doxorubicin and to reduce the translocation, inhibiting the ability of IKK to phosphorylate IκBa. The effect of rapamycin is independent of the blocking of the PI3K / AKT / mTOR pathway, main target of rapamycin, and is reproduced by the depletion of FKBP-51. Moreover, the inhibition of NF-kB leads to an effect of sensitization to apoptosis in melanoma [28].

The same sensitizing effect of rapamycin to drugs of anthracycline family was confirmed in acute lymphoblastic leukemia (cALL) [29]. Rapamycin and doxorubicin showed a cooperative effect likely due to inhibition of NF-kB by FKBP-51 [29]. The interaction between FKBP-51 and NF-kB, responsible for chemoresistance and tumor growth in melanoma and leukemia, has been described also by Jiang and colleagues in glioma cells, in which the growth of tumor cells resulted suppressed by the inhibition of FKBP-51 by RNA interference technique, and induced by overexpression of FKBP-51. In addition, the overexpression of FKBP-51 in glioma cells sensitive to rapamycin resulted in a reduction of the response to the drug. These results confirmed the ability of FKBP-51 to control the NF-kB pathway and the response to rapamycin in glioma cells [34].

1.3.3 FKBP51s: a spliced FKBP5 variant

In 2013, Romano et al [35] showed that in melanoma FKBP51 positively regulates the metastatic potential and the vascular invasion ability of cancer cells. Later, they assessed the levels of FKBP51's transcripts from peripheral blood mononuclear cells (PBMCs) of patients previously operated for a primary melanoma, comparing them with the values found in healthy subjects [36]. In that occasion, they decided to include also the evaluation of the expression of a splice variant of the gene FKBP5. This variant, also called isoform 2 or FKBP51s (short), is codified by the transcript variant 4. The latter has multiple differences in the coding region and 3 'UTR compared to variant 1, because of the occurrence of a frameshift. The protein is shorter because it lacks the TPR domain and has a distinct C-terminus compared to canonical isoform 1. The function of FKBP51s is totally unknown, to date. In the study, the authors postulated that this protein is the result of an interaction between tumor and immune system and could be therefore involved in cancer immunoediting. Surprisingly, an increased expression of the spliced, but not canonical isoform, was detected in PBMC and not in circulating tumor cells (that express high levels of the canonical isoform).

2. Aim

Currently, prognostic and predictive factors able to give indications about the biological aggressiveness of the OSCC are essentially restricted to tumor size and therapy response. To date, oppositely to that occurring in HPV-positive oropharyngeal squamous cell cancer, there are no reliable prognostic or predictive biomarkers for non-oropharyngeal SCC.

As our research group, in the last decade, has identified a relevant role for FKBP51 in tumor aggressiveness, chemo- and radio-therapy resistance in melanoma, and a positive correlation between FKBP51 (full length) expression and poor outcome of OSCC, we now focused our attention on the possible prognostic significance of FKBP51s (short) expression in OSCC.

3. Results

3.1 Population study

3.1.1 Patients' characteristics

Our OSCC series included 49 cases, 20 male and 29 females; the age at diagnosis ranged between 29 and 89 years (mean age 64.5 years, median 66 years). Summary statistic of age at diagnosis is reported in Table 1. Frequency distribution of age at diagnosis in the study population is reported in Figure 1.

Table 1. Summary statistic of age at diagnosis in the study population.

VARIABLE: AGE	
SAMPLE SIZE	**49**
LOWEST VALUE	29,0000
HIGHEST VALUE	89,0000
ARITHMETIC MEAN	64,4694
95% CI FOR THE MEAN	60,3214 to 68,6174
MEDIAN	66,0000
95% CI FOR THE MEDIAN	60,1734 to 72,6532
VARIANCE	208,5459
STANDARD DEVIATION	14,4411
RELATIVE STANDARD DEVIATION	0,2240 (22,40%)
STANDARD ERROR OF THE MEAN	2,0630
COEFFICIENT OF SKEWNESS	-0,7107 (P=0,0400)
COEFFICIENT OF KURTOSIS	0,2586 (P=0,5468)
KOLMOGOROV-SMIRNOV TEST[A]	D=0,0923
FOR NORMAL DISTRIBUTION	accept Normality (P>0.10)

[a] Lilliefors significance correction

PERCENTILES		95% CONFIDENCE INTERVAL
2,5	29,7250	
5	32,8500	
10	45,4000	29,7454 to 54,8372
25	57,0000	50,0922 to 60,8894
75	75,0000	71,2211 to 77,9078
90	80,6000	77,0000 to 87,5091
95	86,0500	
97,5	87,5500	

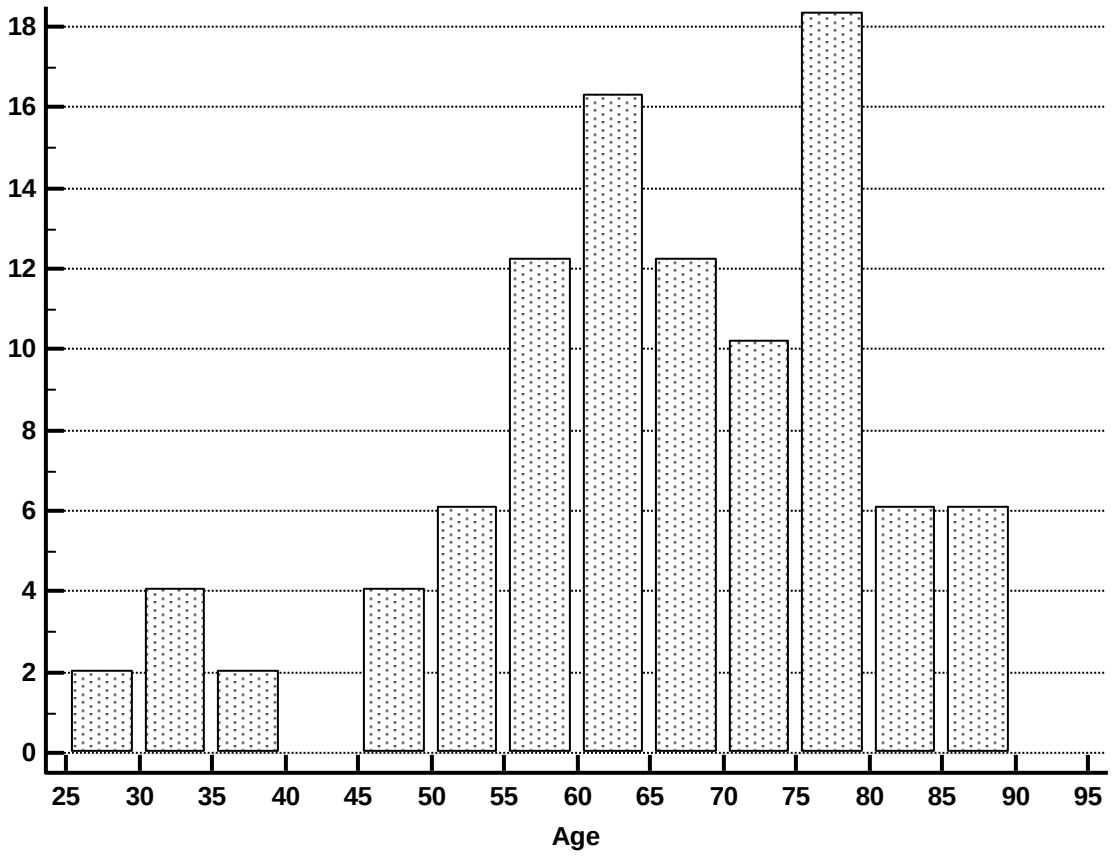

Figure 1. Frequency distribution of Age at diagnosis in the study population.

3.1.2 Tumors characteristics

The observed histotype was SCC for all the tumor under investigation, the most affected site was tongue (51%) (Figure 2).

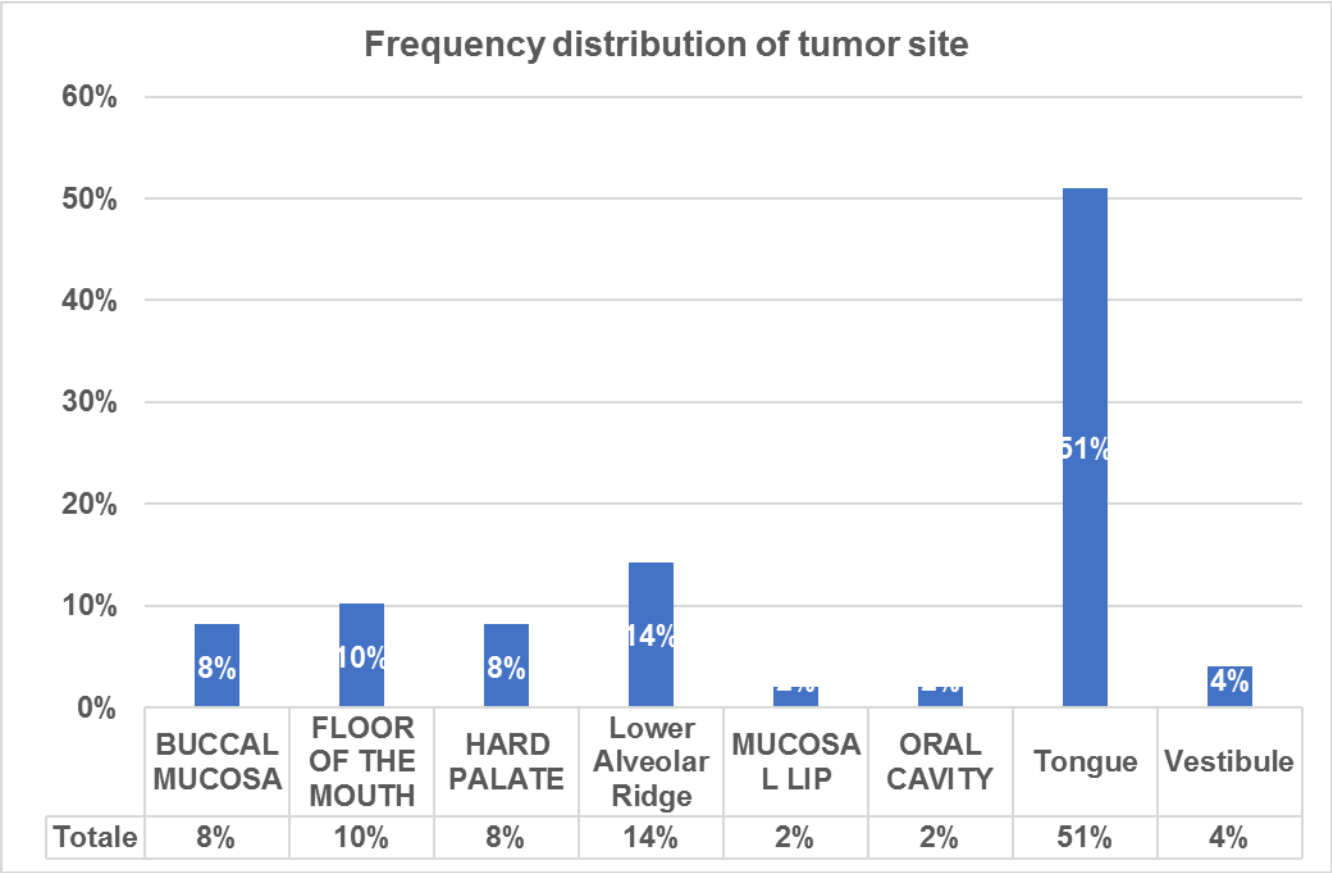

Figure 2. The graph shows the frequency distribution of tumor site in the study population. Most affected site was Tongue (51%).

The pathologic stage of all cancers was determined based on the clinical-pathological information, according to the TNM system [AJCC 7th edition, 12]. 10 out of 49 (20%) samples were classified as pathologic stage I, 12 out of 49 were Stage II (24%), 5 out of 49 were stage III (10%), 21 out of 49 (43%) were Stage IVA, 1 was not assessable (Figure 3).

Based on the histopathological examination, 2 tumors were classified as well differentiated (4%), 18 were moderate (35%), and 29 were poor differentiated (61%) (Figure 4).

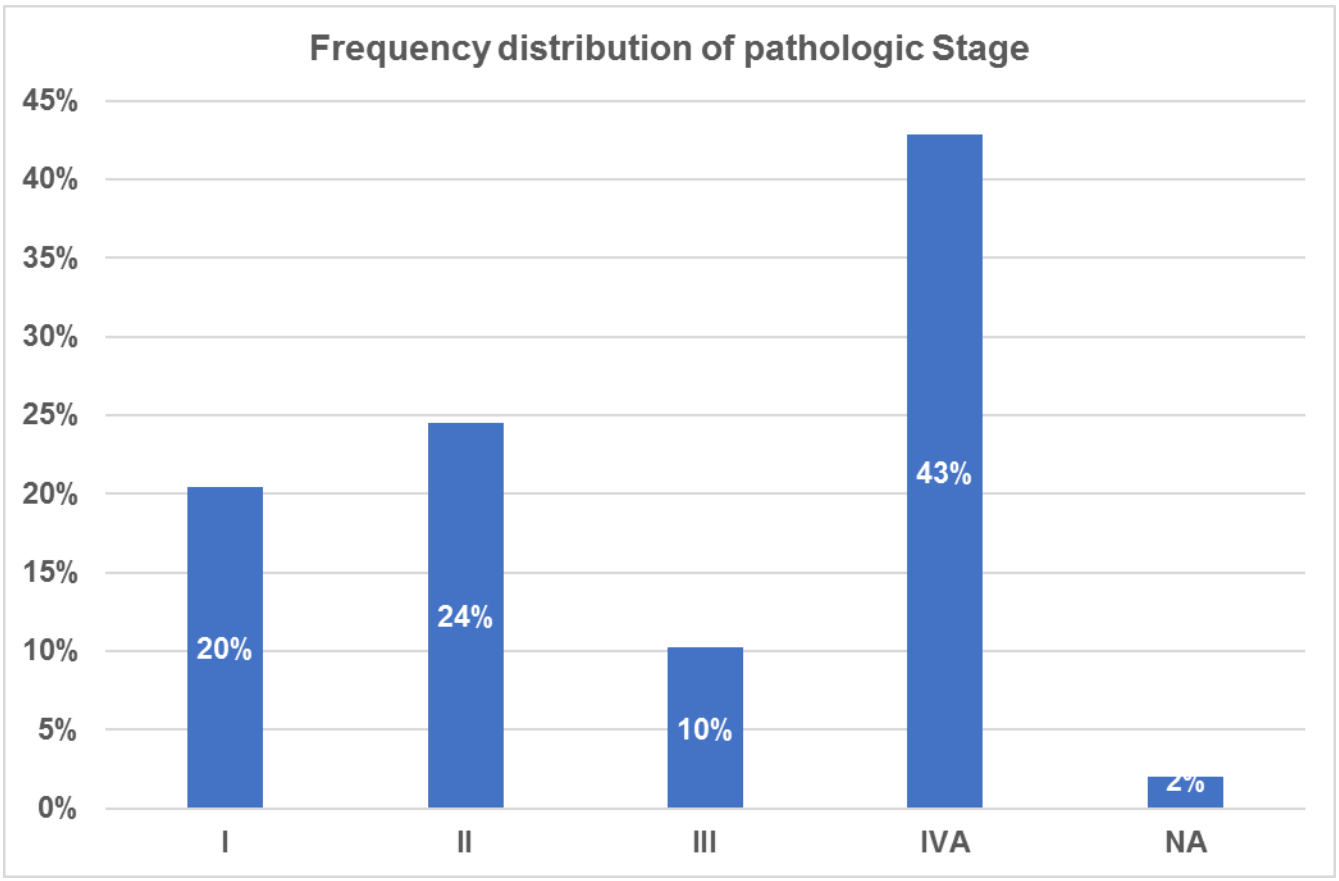

Figure 3. Frequency distribution of Tumor pathologic stages of the study population.

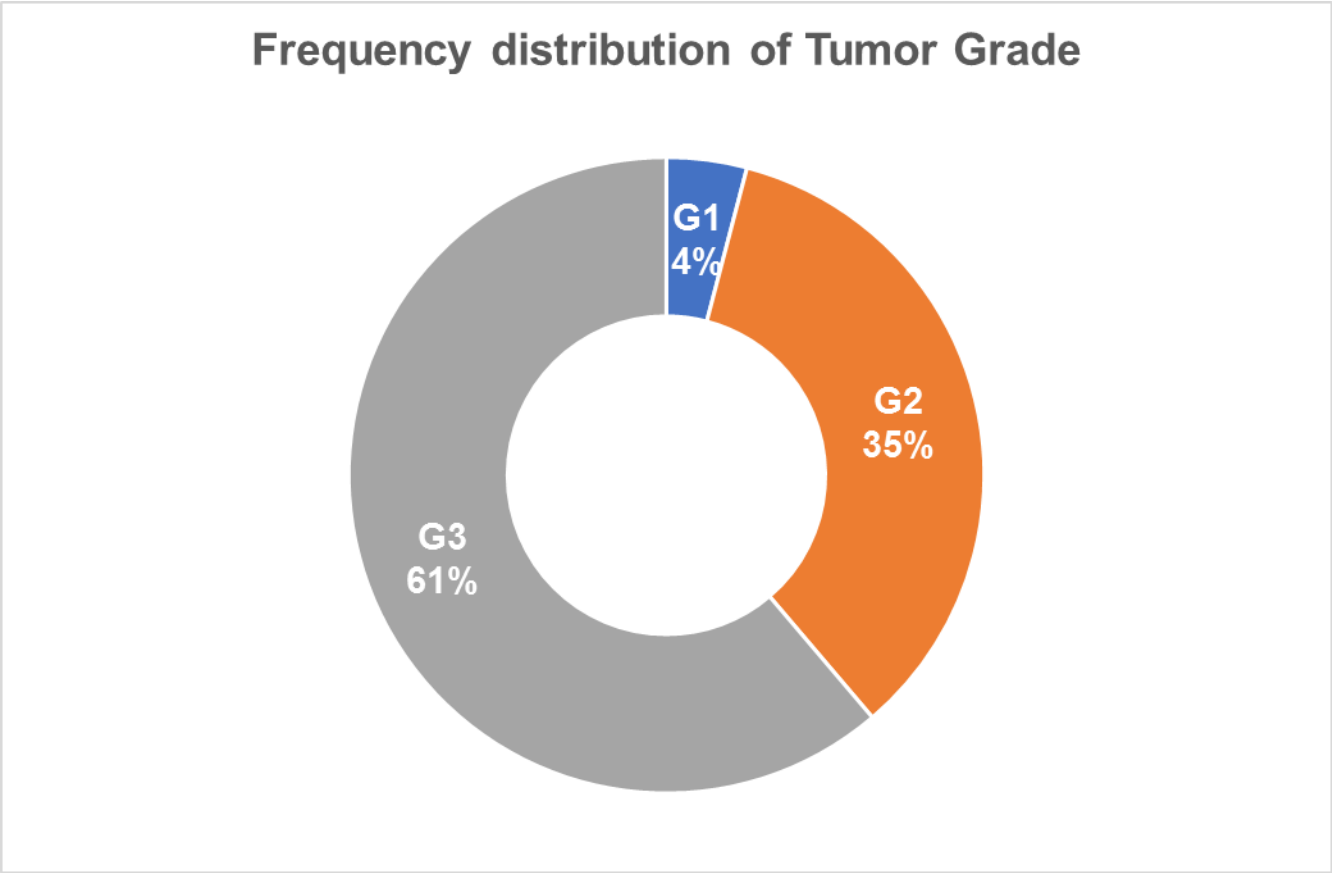

Figure 4. Frequency distribution of tumor grade. 63% of tumors included in the study were poorly differentiated.

During the follow-up period (mean: 38,14 months), 34 out of 49 patients (69.39%) survived and 15 (30,61%) patients died from disease (full follow-up details and all the clinic-pathologic features of the OSCC samples included in the study are showed in Table 2).

Table 2. Full list of OSCC samples included in the study with clinic-pathologic features (NED: not evidence of disease; M: metastasis; R: recurrence; D: death; NA: not assessable).

N°	Sex	Age	SITE	Grade	pTNM	Stage	F-up
1	M	63	TONGUE, LEFT	G1	pT2G1N0	II	131 NED
2	M	54	TONGUE, RIGHT	G1	pT2G2N0	II	94 NED
3	M	61	TONGUE	G2	pT2G1-G2N0	II	38 NED
4	F	29	TONGUE	G2	pT2G2N0	II	67 NED
5	F	33	TONGUE, LEFT	G3	pT2G3N2b	IVA	75 NED
6	M	50	LOWER ALVEOLAR RIDGE, LEFT	G3	pT1G2-3N0	I	32 NED
7	M	75	TONGUE, LEFT BORDER&FLOOR OF THE MOUTH	G3	pT1G2-3N0	I	4,9 R; 69 NED
8	M	77	LOWER ALVEOLAR RIDGE, LEFT	G3	pT2G2-3N0	II	26D
9	M	69	HARD PALATE	G2	pT1G1-2Nx	I	53,60,93R; 93RM96NED
10	M	46	UNDERSURFACE OF THE TONGUE&FLOOR OF THE MOUTH	G3	pT3G2-3N2c	IVA	54NED
11	F	30	TONGUE, RIGHT	G2	pT1G2N0	I	49NED
12	M	58	TONGUE, ANTERIOR	G2	pT2G2N1	III	9M, 16D
13	F	75	ORAL CAVITY	G2	pT4aG2N2b	IVA	82NED
14	M	60	TONGUE, LEFT	G2	pT1G2Nx	I	3R, 29NED
15	F	65	BUCCAL MUCOSA	G3	pT2G2-3N2b	IVA	10R,23M,42NED
16	F	80	TONGUE, RIGHT	G2	pT2G2N0	II	41NED
17	M	60	TONGUE, LEFT	G2	pT2G2Nx	II	3R,11M,30D
18	M	56	FLOOR OF THE MOUTH	G3	pT4aG2-3N2b	IVA	6R,7M,39NED
19	F	69	TONGUE	G3	pT1G2-3Nx	I	38NED
20	F	77	LOWER ALVEOLAR RIDGE, RIGHT	G3	pT1G3N2b	IVA	49D
21	M	75	TONGUE, RIGHT	G3	pT2G3N1	III	29NED
22	F	55	TONGUE, RIGHT	G2	pT2G2Nx	II	28NED
23	M	78	FLOOR OF THE MOUTH	G3	pT4aG2-3N2b	IVA	37D
24	F	77	MUCOSAL LIP	G3	pT4aG2-3N0	IVA	22NED
25	F	68	TONGUE, LEFT	G3	pT1G3N2b	IVA	20NED
26	M	70	FLOOR OF THE MOUTH	G2	pT1G2N2c	IVA	17NED
27	F	35	TONGUE, RIGHT	G2	pT1G2N1	III	84NED
28	F	61	BUCCAL MUCOSA	G3	pT4aG3N0	IVA	13NED
29	F	89	HARD PALATE	G2	pT4aG2Nx	IVA	6R,D
30	F	60	TONGUE, RIGHT BORDER	G3	pT3G3N1	III	24D
31	M	73	FLOOR OF THE MOUTH	G3	pT2G3N0	II	12NED
32	M	45	TONGUE, RIGHT	G3	pT2G2-3N0	II	19R,41D
33	M	76	VESTIBULE LOWER	G3	pT4aG2-3Nx	IVA	53,60,93R,30M,96NED
34	F	57	TONGUE, ANTERIOR	G3	pT2G3N0	II	5,7R; 12D
35	F	74	HARD PALATE	G3	pT4aG3N0	IVA	9M,16NED

36	F	66	BUCCAL MUCOSA	G2	pT1G2Nx	I	29NED
37	F	82	LOWER ALVEOLAR RIDGE	G3	pT4aG3N2b	IVA	12D
38	F	64	VESTIBULE HIGHER RIGHT	G3	pT4aG2-3Nx	IVA	7NED
39	F	67	LOWER ALVEOLAR RIDGE, RIGHT	G2	pT4aG3N2b	IVA	5R,D
40	M	74	LOWER ALVEOLAR RIDGE, RIGHT	G3	G3N0	NA	27NED
41	F	57	TONGUE	G3	pT2G3N1	III	6D
42	F	57	LOWER ALVEOLAR RIDGE, LEFT	G3	pT4aG3N2b	IVA	3R,9D
43	M	61	TONGUE, LEFT BORDER	G3	pT3G3N2b	IVA	9D
44	F	86	HARD PALATE	G3	pT4aG3Nx	IVA	4D
45	F	75	TONGUE, RIGHT	G2	pT1G2N0	I	6NED
46	F	51	TONGUE, LEFT	G3	pT1G3N0	I	2NED
47	F	87	BUCCAL MUCOSA	G3	pT1G3N0	I	9 R, 11 NED
48	F	81	TONGUE, RIGHT	G2	pT2G1-2N0	II	4NED
49	F	71	FLOOR OF THE MOUTH	G3	pT4aG3Nx	IVA	30M

3.2 Expression analysis by immunohistochemistry

3.2.1 FKBP51s immunohistochemical expression

The study of tissue FKBP51short protein expression has been conducted using immunohistochemical staining [36]. Protein expression levels were reported for each sample, with a two-tied score for the tumor expression. In the definition of score were considered separately the intensity of staining and the percentage of positive cells.

As for the intracellular localization in all 49 cases of squamous cell carcinoma of the oral cavity analyzed, regardless of the intensity of staining, FKBP51short was always observed in the cytoplasm of positive cells. From a topographical point of view, the overexpression of the protein resulted restricted to the invasion front. Only in a few cases, we have observed a clear and convincing positivity even in intratumoral portion.

3.3 Data analysis

From analysis of the data and the correlation test with clinic-pathological parameters of each sample, we observed a clear separation of the study population, including negative tumors or poorly expressing FKBP51short (<5% of cancer cells, regardless of staining' intensity) on the invasion front and tumors with a high expression (> 50% of tumor cells, with a judged moderate / high intensity), on the invasion front. On the basis of these criteria, and through the correlation analysis, we identified two clusters of tumors; 29% (14/49) of

OSCC showed poorly expression of protein on the invasion front, the remaining 71% (35/49) showed, however, a strong and diffuse positivity on the invasion front. (Figure 5, 6)

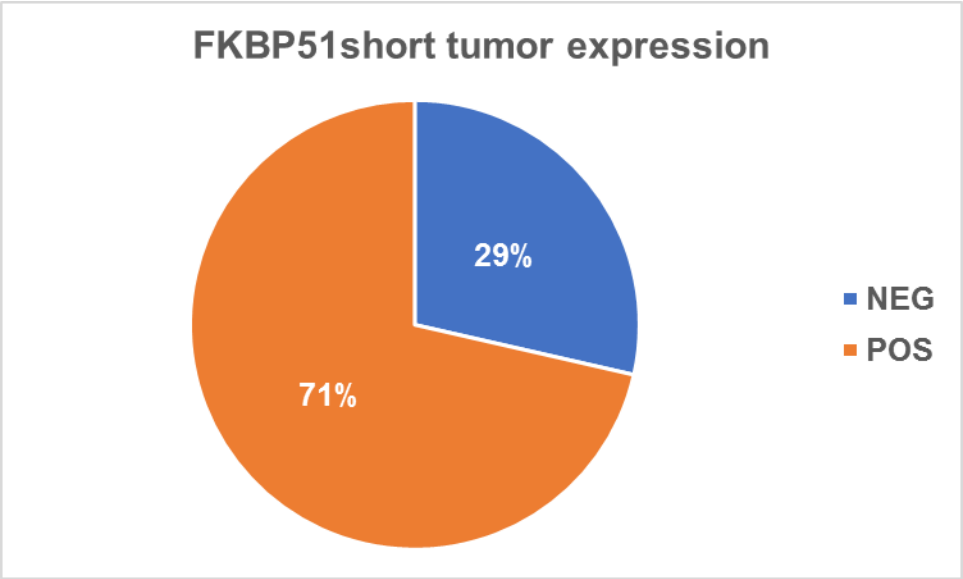

Figure 3. The tumor series could be separated in two clusters, according to FKBP51s tumor expression. 29% (14/49) of OSCC showed poorly expression of protein on the invasion front, the remaining 71% (35/49) showed a strong and diffuse positivity in terms of invasion front.

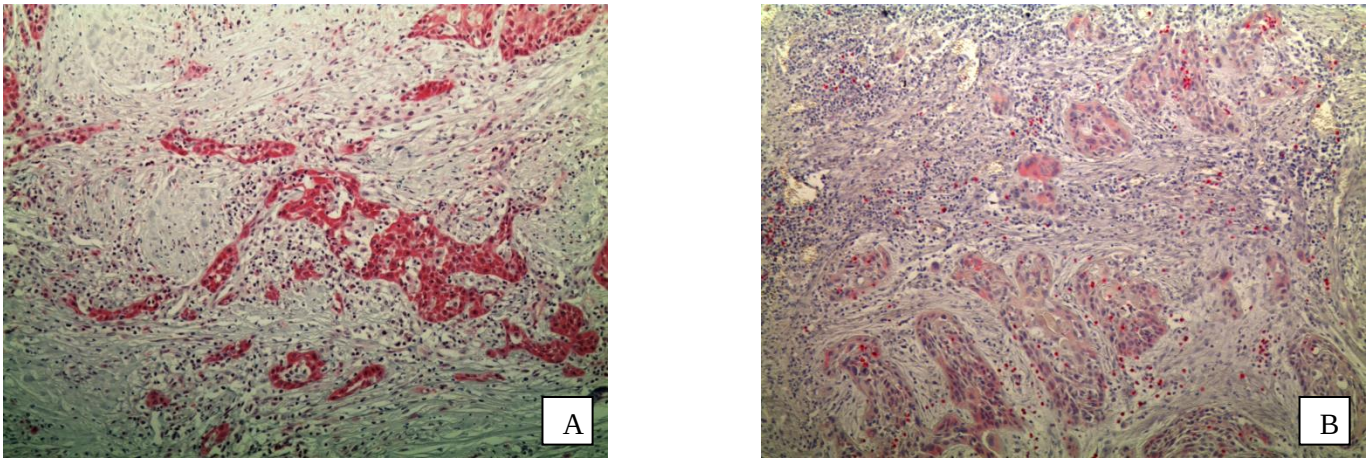

Figure 6. A case of OSCC with a strong and diffuse positivity in terms of invasion front (A) and a case with poorly expression of protein on the invasion front (B).

Analyzing survival data, 69% (34/49) patients with OSCC showed favorable outcome, while 31% (15/49) showed poor outcome. (Figure 7)

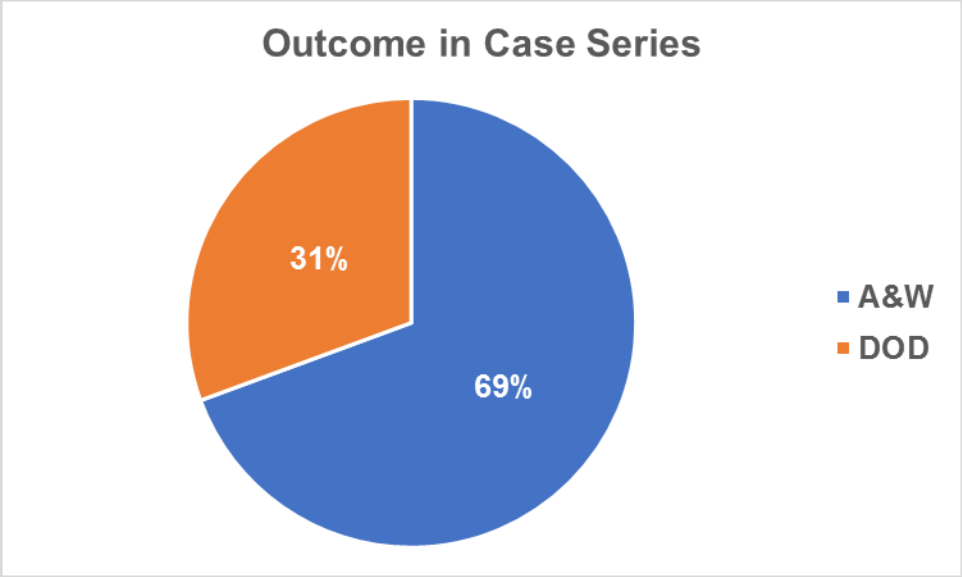

Figure 7. Outcome distribution in the Case Series, relatively to Overall Survival parameter. (A&W: alive and well; DOD: dead of disease).

Comparing the data concerning FKBP51short expression with disease outcomes, we observed that 93.3% (14/15) of cases with poor outcome showed significantly strong positivity for FKBP51short on the invasion front, opposed to the 6.7% (1/15) of cases with focal or low expression of the protein. (Figure 8)

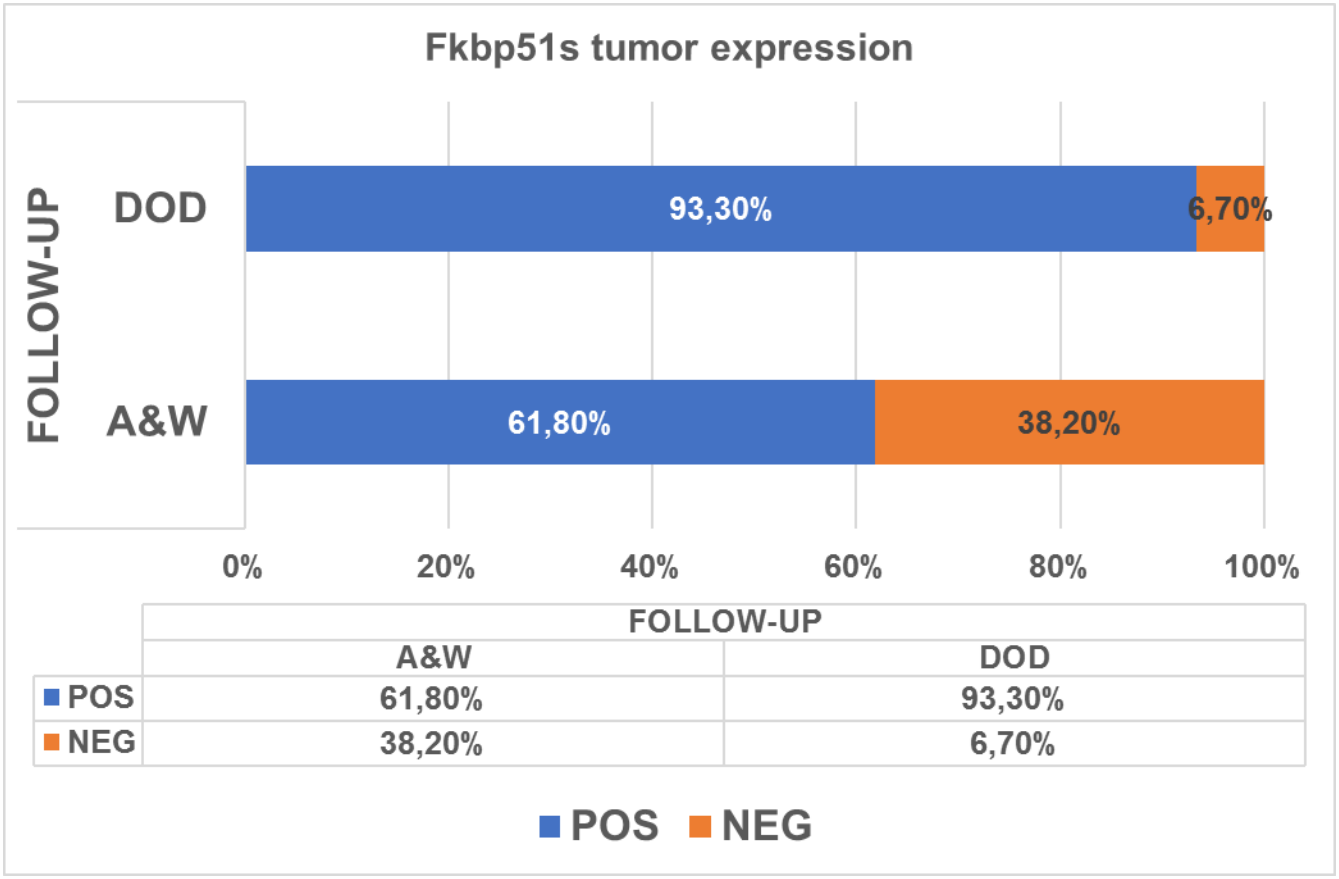

Figure 8. Chart showing the frequency distribution of FKBP51short Positive and Negative tumors between the two F-UP categories DOD (dead of disease) and A&W (alive and well).

Table 3. Statistical table showing FKBP51s figures distribution between the two follow-up categories (overall survival).

	DOD	**A&W**	*TOT*
FKBP51s POS	14	21	35
FKBP51s NEG	1	13	14
TOT	15	34	49

Table 3 summarizes the FKBP51s figures distribution between the two follow-up categories, in terms of overall survival (see also Figure 9).

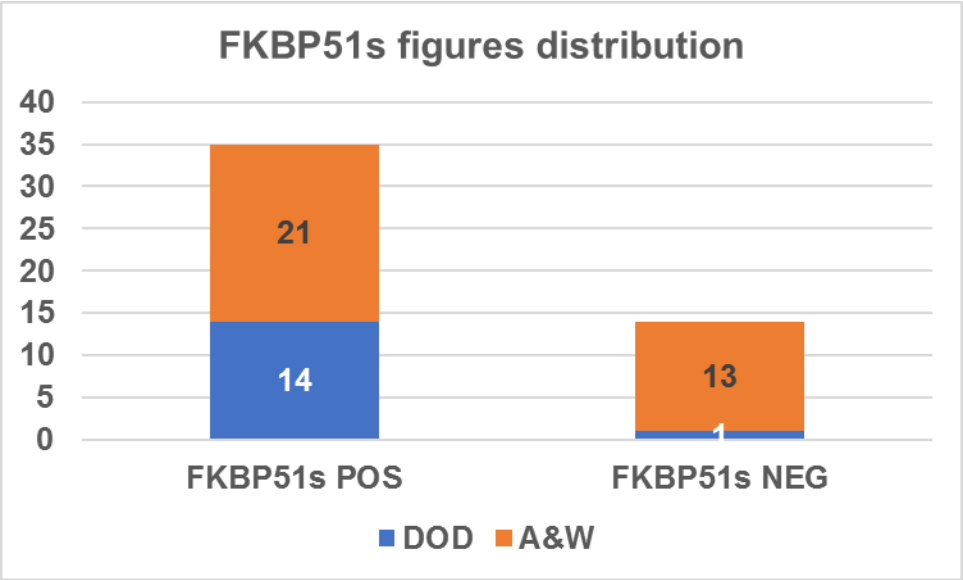

Figure 9. The chart shows FKBP51s tumor expression figures distribution between the two follow-up categories.

The tumors with strong positivity for FKBP51short on the invasion front (35 cases among OSCC) showed an absolute risk (AR) of death due to cancer equal to 40% (14/35), while negative or poorly expressing tumors showed an AR of 7% (1/14) (Figure 10); the Odds of death were 67% for tumors with FKBP51short + versus 8% for tumors with FKBP51short – (Figure 11 b). Conversely, The Odds to get a good outcome were 130% for FKBP51short negative tumors (Figure 11b).

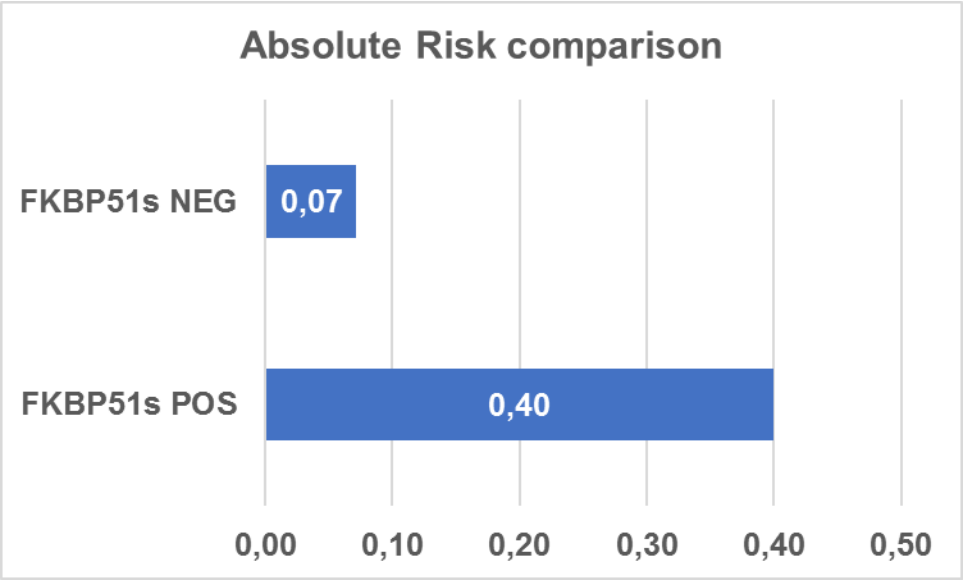

Figure 10. The chart shows the Absolute Risk of Death comparison between Negative and Positive Fkbp51s tumor expression categories.

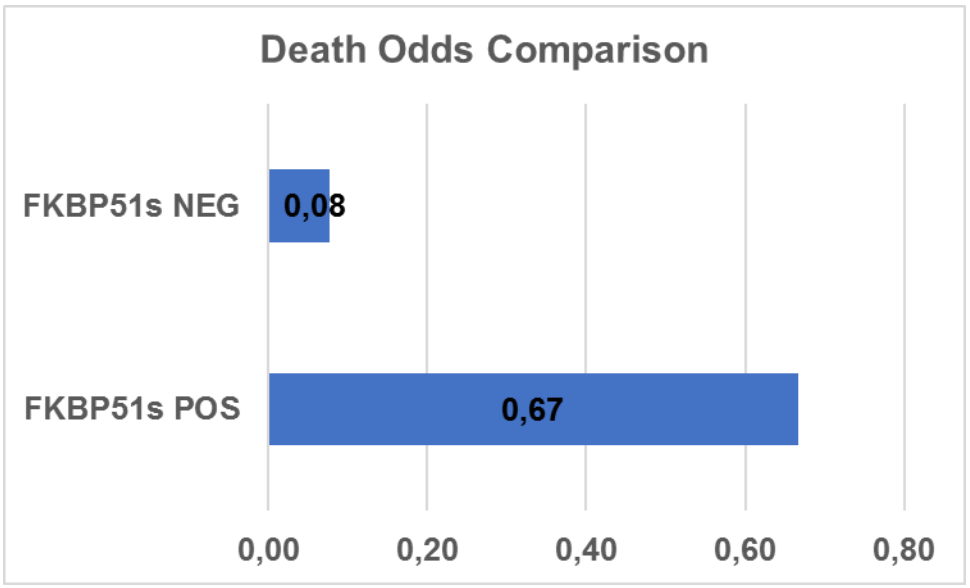

Figure 11a: Odds of Death are significantly higher for FKBP51s Positive category.

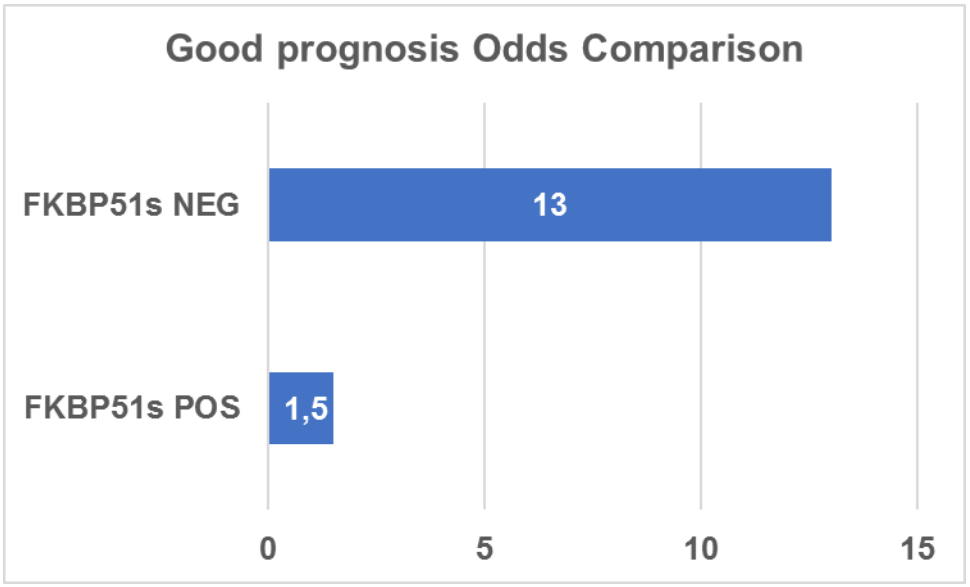

Figure 4b. Poor FKBP51s tumor expression significantly increases the Odds of a good prognosis at the end of the follow up.

In conclusion, the Relative Risk (RR) of death for FKBP51short+ tumors was 20 times the one of negative tumors. The Odds Ratio (OR) between positive and negative tumors, relatively event of death, was equal to 9.57. Our risk and Odds analysis were statistical significant with p values <0.05.

Survival curves analysis (Table 4) about FKBP51short+ and FKBP51short- on the invasion front, showed that FKBP51short+ tumor patients had a mean survival of about 58 months, compared with the average survival of

patients with FKBP51short- tumors of approximately 121 months; the mean overall survival of our OSCC patients was 86 months (Table 5).

The Hazard Ratio of FKBP51short Positive patients was about 5.8 time the FKBP51short negative ones (Table 6).

The differences between the two survival curves was statistically significant at the Log-rank test (p = 0.05) (Figure 12).

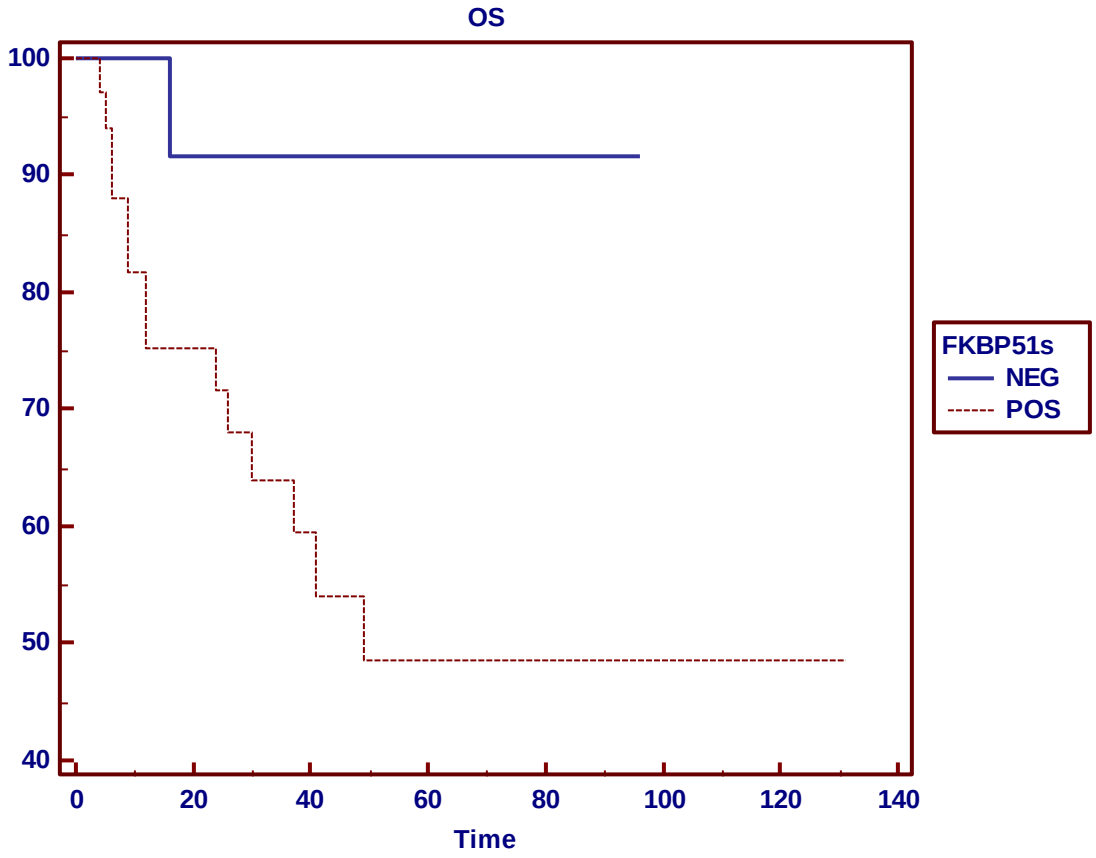

Figure 5. Survival Curves according to Kaplan Meier, showing a good separation between the FKBP51s Negative and Positive curves.

Table 4. Cases summary for survival analysis.

Factor	Number of events		Number censored		Total sample size
	N	%	N	%	
NEG	1	7,14	13	92,86	14
POS	14	40,00	21	60,00	35
Overall	15	30,61	34	69,39	49

Table 5. Survival analysis: mean survival with 95% CI for the mean.

Factor	Mean	SE	95% CI for the mean
NEG	121,417	9,175	103,433 to 139,400
POS	58,193	7,393	43,702 to 72,684
Overall	85,523	9,463	66,976 to 104,070

Table 6. Hazard ratio (column/rows) with 95% Confidence intervals.

Factor	NEG	POS
NEG	-	5,7645 1,8933 to 17,5511
POS	0,1735 0,05698 to 0,5282	-

In order to confirm the association between the tissue expression of FKBP51short and tumor invasiveness, we tested the immunohistochemistry expression of the protein also on a series of pre-neoplastic lesions, carcinoma in situ and micro invasive carcinomas.

The pre-neoplastic lesions (with medium degree dysplasia) were found negative for FKBP51s, in the case of severe dysplasia and in situ carcinomas an exclusively nuclear staining was observed, which shifted toward cytoplasm exclusively in micro invasive areas.

3.4 Apoptosis analysis

The appearance of a hypodiploid peak (sub G1) revealed cells with fragmented DNA. As shown in Figure 13, the cells treated with rapamycin undergone cell death. Rapamycin induced death in 32.5 + 6.9% of the cultured cells ($p < 0.006$, $N = 4$). In Figure 13 we show representative histograms of the DNA content of untreated or cells cultured with rapamycin (100 ng/ml for 24h). The percentage of apoptotic cells (hypodiploid) is indicated on the bar. The cytotoxic effect of rapamycin suggested that FKBP51 supports the survival of tumor cells. We therefore used more selective inhibitors of FKBP51 [37] defined FKBP51 inhib # 1 and 2, together with a structural analogue- not inhibitor (control drug). The apoptosis texts revealed an apoptotic effect of selective inhibitors, that although it is less marked than that of rapamycin, however, remains significant (Figure 14).

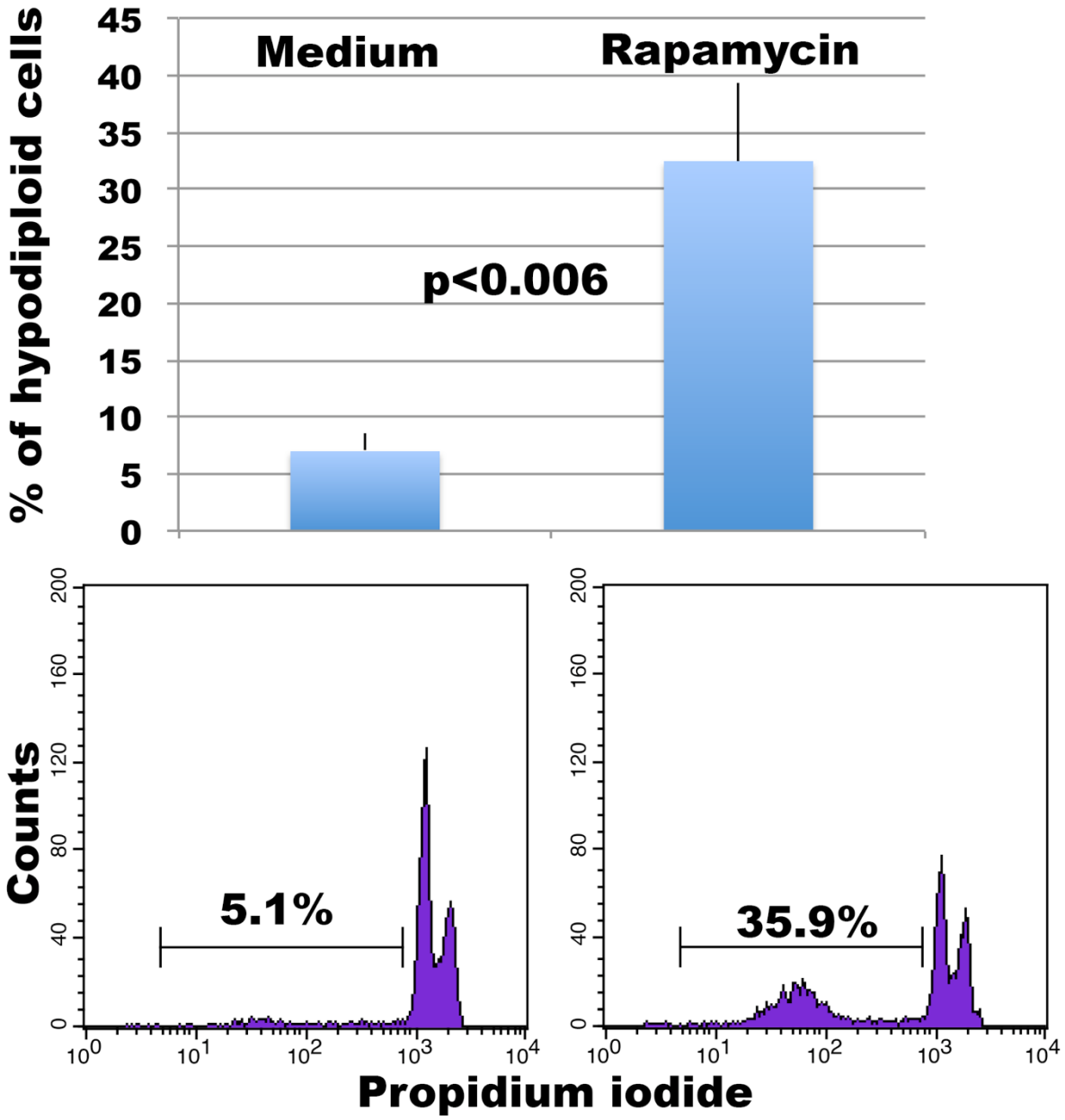

Figure 6. Top, Graph representing means and standard deviations (N=4) of hypodiploid cell percentage of KB cell stimulated or not with rapamycin for 24h. Bottom, representative flow cytometric histograms of the propidium iodide incorporation of KB cells, bars indicate the percentage of cell in sub G0/G1 phase, namely apoptotic cells.

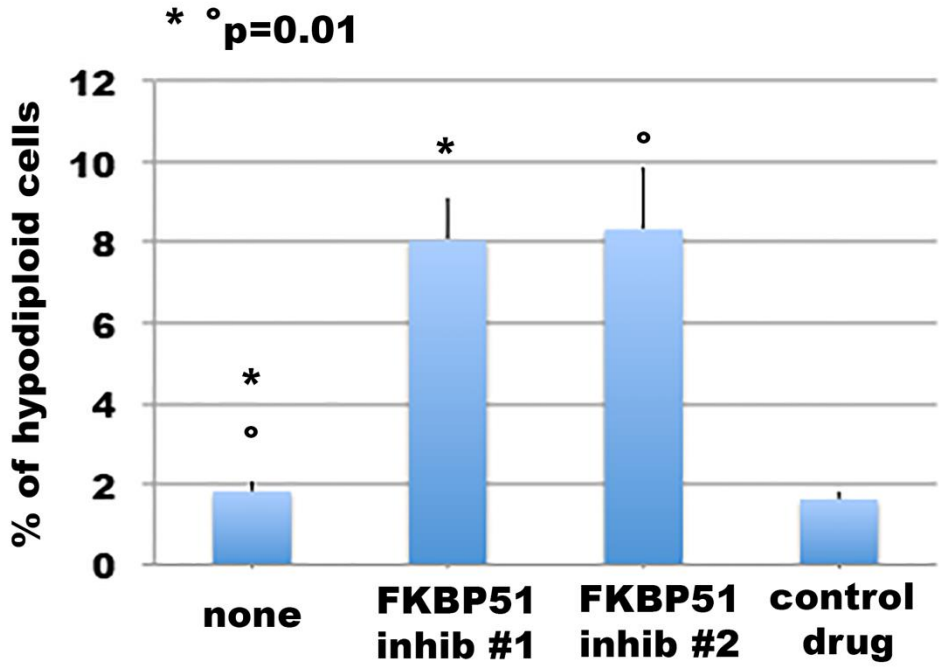

Figure 7. Graph representing means and standard deviations (N=4) of hypodiploid cell percentage of KB cell stimulated or not with two different inhibitors specific for FKBP51 (#1 and 2) and a control drug as reference sample, for 24h.

4. Materials and Methods

4.1 Patients and tissue samples

Formalin-fixed, paraffin-embedded tissue blocks of 49 primary OSCCs, diagnosed and excised with healthy surgical margins from January 2000 to December 2013, were retrieved from the archives of the Pathology Section of the Department of Advanced Biomedical Sciences, 'Federico II' University of Naples. The clinical data and pathological features of the tumors are reported in Table 2. No patient experienced radiotherapy before surgery. The study design and procedures involving tissue samples collection and handling were performed according to the Declaration of Helsinki, in agreement with the current Italian law, and to the Institutional Ethical Committee guidelines.

4.2 Immunohistochemistry

For each case, we selected a block of tissue fixed in formalin and embedded in paraffin representative of the tumor, and used it to obtain serial sections. One section was stained with hematoxylin / eosin to confirm the initial diagnosis; the others were used for immunohistochemical investigation.

Immunohistochemical analysis was performed on 4-μm thick serial sections, mounted on poly-L-lysine coated glass slides. The sections were deparaffinized and subjected to antigen retrieval by microwave oven treatment (3 min x 3 times, in TRIS-EDTA-CITRATE buffer, pH 7.8); the backdrop (for blocking non-specific background staining) was removed using the universal blocking serum (Dako Diagnostics, Glostrup, Denmark) for 15 min at room temperature. Endogenous alkaline phosphatase activity were quenched adding Levamisole to buffer AP (Substrate Buffer); the slides were rinsed with TRIS+Tween20 pH 7,4 buffer, and incubated in a humidified chamber with the primary antibody anti-FKBPshort (rabbit polyclonal handmade antibody, diluted 1:100 1 h at room temperature). Then used a biotinylated secondary antibody and streptavidin conjugated with alkaline phosphatase. The reaction has been highlighted with the chromogen fast red, which showed the presence of antigen that we sought in red (Dako REAL Detection System, Alkaline Phosphatase / RED, Rabbit / Mouse). Again, after a weak nuclear counterstain with hematoxylin, the sections were then mounted with a synthetic medium (Entellan, Merck, Darmstadt, Germany).

4.3 Analysis of apoptosis

Analysis of DNA content by propidium iodide incorporation was performed in permeabilized cells by flow cytometry. KB cells (2x104) were stimulated with rapamycin 100ng/ml, the FKBP51 inhibitors 1 and 2 40nM,

or a control compound which resulted to have no effects on FKBP51. Cell was harvested 24 h after stimulation, washed in PBS, and resuspended in 500 μl of a solution containing 0.1% sodium citrate w/v, 0.1% Triton X-100 v/v, and 50 μg/ml propidium iodide (Sigma Chemical Co, Gallarate, Italy). After incubation at 4°C for 30 min in the dark, cell nuclei were analyzed with a FACScan flow cytometer. Cellular debris was excluded from the analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of the elements in the hypodiploid region was calculated.

NB: This experiment was carried out thanks to Prof. M.F. Romano and Dr. S. Romano, Department of Molecular Medicine and Medical Biotechnology, University "Federico II", Naples, Italy.

4.4 Image acquisition

Histological figures have been acquired by a Leica DFC290 microscope, at highest resolution and saved in TIFF. Then, images have been assembled by Adobe Photoshop CS.

4.5 Statistical analysis

The statistical analysis was performed using the IBM SPSS software.

The correlation between expression of FKBP protein and OS was validated by contingency tables, using the chi-square.

The calculation of RA, RR, and OR was conducted according to Altman method [38].

Patients were stratified according to the risk of death at the follow-up by Kaplan-Meier survival curves analysis. For comparison of the event-free survival time (events: death) between two categories of individuals, the log-rank Mantel–Haenszel test was applied.

For all analyses, a p value of .05 or less was considered significant.

Evaluation of the intraobserver agreement for the tested proteins on whole sections was performed by use of Cohen's weighted kappa statistic.

5. Conclusions and Discussion

5.1 The scheduled expression of FKBP51s (short) form and its overexpression as identification as prognostic biomarker for OSCC.

In previous studies, we demonstrated the essential role played by FKBP-51 in the activation of NF-kB induced by ionizing radiation and the consequent radioresistance of skin melanoma, indicating that the immunophilin may be regarded as a possible new molecular target for personalized therapeutic strategies [30].

In particular, we have identified a relevant role in chemo- [29] and radio-therapy [30] resistance for the large immunophilin FK506 binding protein 51 (FKBP51), revealing for the first time an association between FKBP51 expression, as determined by immunohistochemistry, and tumor aggressiveness [30].

In >70% of our cases, melanoma cells in the radial growth phase showed a low immunopositivity for FKBP51, whereas a strong signal was detected in vertical growth phase, with the highest immunoreactivity found in the metastatic melanoma cases. This prompted us to postulate a definite role for FKBP51 as a novel marker of melanocyte malignancy. [39]. For the first time, in this paper we also reported the expression of FKBP51 in HNSCC. Our preliminary observations strongly suggested that the overexpression of FKBP51 may be considered a promising hallmark of malignancy and resistance to therapy in HNSCC. We completed and expanded the study on a series of OSCC by immunohistochemistry. Finally, given an IHC-test resulting, we quantified the risk of a poor outcome for the FKBP51high phenotype OSCC, calculating the probability of a patient death occurrence. Our study supported the conclusion that a positive correlation between FKBP51 expression and the poor outcome of OSCC exhists [40]. Recently, FKBP51-selective compounds, SAFit1 and SAFit2, has been shown to effectively inhibit FKBP51 different functions, especially those pro-cancer, through suppression of NF-kB signal [41-42]. Future studies are needed to verify if these compounds in oral cancer, in combination therapy, may improve treatment outcomes.

In the present study, we found that most of the OSCC cases expressed also FKBP51s. The highest levels of protein expression have been found only at the invasive front of the tumors (Figure 15). The distinct cytoplasmic positivity for FKBP51s at this tumor subsite correlated, in our series of OSCC, with poor prognosis with a significantly reduced median survival. Comparing these results with the expression of the protein in pre-neoplastic lesions and in situ and / or micro invasive carcinomas, we concluded that the cytoplasmic expression of FKBP51s may have a role in the mechanisms of invasion and migration of cancer cells, with the shift of the signal for FKBP51s from the nucleus (carcinoma in situ, figure 16) to the cytoplasm (invasive carcinoma) appearing to be a key step toward the acquisition of the invasive phenotype.

In order to confirm our first observation concerning the positive correlation between FKBP51 expression and the poor outcome of OSCC [40] we performed, in this study, apoptosis tests in KB cells (squamous cell carcinoma

cell line), treating the cells with rapamycin, an inhibitor of FKBP51, and more selective inhibitors of FKBP51 (FKBP51 inhib # 1 and 2). The tests revealed an apoptotic effect of selective inhibitors, confirming the role of FKBP51 in supporting the aggressive phenotype and tumor resistance of OSCC, and suggesting that FKBP51 could be a candidate target for innovative therapies. In particular, based on our observation about the scheduled expression of FKBP51s already in lesions with severe dysplasia and its dynamic shift in the cytoplasm of invasive lesions, the use of selective inhibitors of FKBP51 may be effective and extend the spectrum of standard therapies.

As a complementary step, we explored The Cancer Genome Atlas (TCGA) screening two published series relating to 520 (online only) and 279 cases of HNSCCs [43]. We analyzed the expression pattern of genes related to FKBP51 as possible with the data processing software provided by the website. Among the most frequently genes, we found Tensin 4, which codes for a protein involved in cell migration mechanisms, binding of actin filaments, ADORA 3, encoding a protein receptor of adenosine, described in the mechanisms of proliferation and cell death, and ZBTB16, coding for a zinc finger transcription factor involved in cell progression mechanisms.

The presence of such genes, involved in cell migration, supports our hypothesis that the expression of FKBP51s in cytoplasm of cells of the invading front of OSCC could be associated to the acquisition of the invasive phenotype. This evidence is fully in agreement with the poor prognosis of the OSCC of our series overexpressing FKBP51s. To our knowledge, this is the first report about this phenomenon.

These results support the hypothesis of a role for either FKBP51 and FKBP51s as useful prognostic markers, easily detectable by immunohistochemistry at the time of the first diagnosis. They can significantly contribute to the better stratification of patients with OSCC in more defined risk categories allowing them to benefit from new target therapies. Our results lead us to postulate that the introduction of selective FKBP51 inhibitors (either of FKBP51 and FKBP51s) may contribute, in the next future, to the reduction of the still discouraging mortality rate of patients with OSCC.

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