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MORPHOLOGICAL AND MORPHOMETRIC ANALYSIS OF CUTANEOUS SQUAMOUS CELL CARCINOMA IN PATIENTS WITH RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA: A RETROSPECTIVE STUDY

ABSTRACT

Background: Recessive dystrophic epidermolysis bullosa is a highly disabling genodermatosis characterized by skin and mucosal fragility and blistering. Cutaneous squamous cell carcinoma (cSCC) is one of the most devastating complications, having a high morbidity and mortality rate. Patients with recessive dystrophic epidermolysis bullosa were reported to have up to a 70-fold higher risk of developing cSCC than unaffected individuals. Immune cells play a role in cancer evolution.

Objective: The aim of our study is to evaluate immuno-histological differences between cSCC in patients with and without recessive dystrophic epidermolysis bullosa.

Methods: A retrospective study of 25 consecutive cases was performed; 5 were biopsies of cSCC taken from 5 patients with recessive dystrophic epidermolysis bullosa; as controls we analysed 10 cSCC in subjects without recessive dystrophic epidermolysis bullosa (5 primitive, 3 post-burns and 2 post-radiotherapy), 5 cSCC in renal transplant recipients and 5 cutaneous pseudoepitheliomatous hyperplasia in patients with recessive dystrophic epidermolysis bullosa.

Results: A significant reduction of CD3+,CD4+,CD68+ between the cSCC in patients with recessive dystrophic epidermolysis bullosa compared to primary cSCC and a significant reduction of CD3+, CD4+, CD8+, CD20+ was observed in cSCC in patients with recessive dystrophic epidermolysis bullosa compared to secondary cSCC. On the contrary there was no difference in CD3+, CD8+, CD20+, CD68+ expression when comparing cSCC in patients with recessive dystrophic epidermolysis bullosa to cSCC in renal transplant recipients .

No significant difference was found in size, histopathology, grading, number of mitoses and EGFR expression between the different groups.

Conclusions: Our data show a reduction in immune cell peritumoral infiltration. Considering the well-known evolution of cSCC in patients with recessive dystrophic epidermolysis bullosa, as well

as the younger age at diagnosis, it can be assumed that immune dysfunction might contribute to the cSCC aggressiveness in these patients.

INTRODUCTION:

Recessive dystrophic epidermolysis bullosa (RDEB) is a genodermatosis characterized by an increased fragility of skin and mucosa and the formation of blisters. [1] This form is caused by premature stop codons in the COL7A1 gene transcript, that yields smaller than normal collagen VII proteins that are unable to guarantee anchorage between epidermis and dermis . [2-3-4]

Among the disease complications, there is an increased risk of an aggressive form of cutaneous squamous cell carcinoma (cSCC). The mean age at diagnosis of this tumor in epidermolysis bullosa patients is approximately 36 years (range 27-48 years), whereas it is generally diagnosed at about 80 years (range 73-86 years) [5] in non epidermolysis bullosa subjects. Adult cSCC-RDEB patients die within 5 years due to metastatic cSCC despite aggressive surgical resection. [6]

In RDEB patients the cumulative risk ranges from 7.5% at 20 years to 90.1% at the age of 55, and the mortality risk from 38.7% at the age of 35 to 78.7% by the age of 55.[6]

The mechanism through which the lack of collagen VII is correlated with cSCC has been the focus of in-depth study. A key role seems undoubtedly to be played by the accumulation of genetic and epigenetic hits, that transform the epithelial cells into neoplastic cells.[7]

The aggressive biological behaviour could also depend on the marked malnutrition, [8] reduced Natural Killer (NK) cell surveillance of the tumoral cells,[9] the p53 mutation, [10] and the innate immune deficiency correlated to the lack of collagen VII.[11]

Considering these hypotheses, it is very important to gain a better understanding of both the cellular and the mesenchymal mechanisms that induce the onset of cSCC in RDEB patients.

A retrospective observational study was conducted to assess the histological and immunohistochemical (IHC) differences in cSCC in REDB patients versus non REDB subjects.

MATERIALS AND METHODS:

A retrospective study of 25 consecutive cases was performed. We reviewed 5 cases of cSCC in 5 RDEB patients and compared them with 10 consecutive cases of cSCC in non RDEB subjects, 5 primitive and 5 secondary cSCC (3 post-burns and 2 post-radiotherapy); 5 cSCC in kidney organ transplant recipients) (OTR); 5 cutaneous pseudoepitheliomatous hyperplasia in patients affected by RDEB were also included in the study as controls. Thus, 5 groups were studied: RDEB cSCC; primitive cSCC; secondary cSCC, cutaneous pseudoepitheliomatous hyperplasia on RDEB skin, 5

kidney OTR cSCC. The kidney OTR cSCC patients received post transplant immunosuppressive therapy as a combination of steroids (Prednisone) associated to calcineurin inhibitors (3 with Cyclosporin A and 2 with Tacrolimus) and mycophenolate mofetil.

In each case, gender, age, skin lesion site, size (largest diameter of the lesion) were recorded. In addition, in neoplastic biopsies, particular attention was paid to the cSCC histological subtype (classic variant, acantholytic, sarcomatoid, pseudovascular, adenosquamocellular, verrucous), grading (well-differentiated G1; moderately-differentiated G2; poorly-differentiated G3) and number of atypical mitoses (per field).

Classification of the site of the cSCC was based on the National Comprehensive Cancer Network (NCCN) Classification 2017. [12]

The histological tissues obtained by excisional skin biopsy were fixed in neutral buffered formalin at pH 7, dehydrated, paraffin-embedded and stained with haematoxylin eosin.

From the paraffin-embedded blocks, 5 µm thick sections were de-paraffinized, hydrated and subjected to immunohistochemical analyses using the following antibodies:

Anti-CD3: mouse monoclonal Ab (mAb), code NCL-L-CD3-565, (Novocastra laboratories LdT.), diluted 1:200; Anti-CD4: mouse monoclonal Ab (mAb),code M7310,(DAKO ,Carpinteria,California,USA),diluted 1:50; Anti-CD8: mouse monoclonal Ab (mAb),code NCL-CD8-295,(Novacastra Laboratories LdT.),diluted 1:50 ; Anti-CD20 : mouse monoclonal Ab (mAb) ,code M0755,(DAKO ,Carpinteria,California,USA),diluted 1:400; Anti-CD68 : mouse monoclonal Ab (mAb), code M0876,(DAKO ,Carpinteria,California,USA),diluted 1:100; Anti-EGFR: mouse monoclonal Ab (mAb),code M7239,(DAKO ,Carpinteria,California,USA),diluted 1:4; Anti-Foxp3: mouse monoclonal Ab (mAb),code236A/E7,eBioscience,diluited 1:100. Anti-PDL-1:mouse monoclonal Ab (mAb), code M3653,(DAKO, Carpinteria,California,USA),diluted 1:50.

The blocks were pre-treated with the PT-LINK device (DAKO), using EDTA (EnVision Flex, target retrieval solution, High Ph(50x), DAKO) for antibodies CD3, CD4, CD8, CD68, FoxP3 and Citrate (EnVision Flex, target retrieval solution, Low Ph(50x), DAKO) for antibodies CD20, PDL-1 and EGFR.

The IHC reaction was obtained with the Autostainer Link 48 device (DAKO), evaluating in each clinical case the cell density (number of positive cells in 10 microscopic fields), using the Reichert Polyvar 2 microscope, with a digital telecamera JTV and Trinitron monitor, Sony. The positive cells were manually quantified. Each field was examined at 400 X magnification; the fields were 140 µm long by 110 µm wide, and the total field area was 15.400 μ^2. The fields examined were randomly chosen.

EGFR expression was assessed on the chromogen signal on the tumoral cell membrane, on a scale of 4 (score 0: no expression; score 1: a weak, inconstant signal; score 2: inconstant, discontinuous expression; score 3: continuous membrane expression on most of the tumoral cells).

PDL-1 expression was scored [0 as negative (<1% stained cells);1 very low (≥1% to <10%);2 low(≥10% to <25%);3 intermediate (≥25% to <50%) ;4 high (≥50%)].

Statistical Analysis

For each patient, mean and standard deviation values for the 10 fields were recorded. A normal distribution of values was checked using the Kolmogorov-Smirnov test. When non significant results emerged, the values were considered normally distributed and subjected to parametric tests; otherwise, non parametric tests were applied.

Comparison was made of the means within the single groups and in the five study groups (RDEB cSCC, primitive cSCC, secondary cSCC, RDEB cutaneous pseudoepitheliomatous hyperplasia, kidney OTR cSCC). Atypical mitoses, EGFR expression were non parametric values and analysis of the means was carried out by Mann-Whitney test. The CD3, CD4, CD8, CD20, CD68, and FoxP3 values, as well as the age of onset, were parametric and analysed by Student's T test.

Contingency tables were also applied, and Chi-square tests were made of the clinical-histological data (gender, lesion site, histological grade and histological subtype). We applied the ANOVA Test for age and lesion size. Significance was set at $p < 0.05$.

All histological analyses were carried out using the Prism 6 program, GraphPad software, La Jolla (USA).

RESULTS:

Gender, age, lesion site, size, histological subtype, differentiation grade and number of atypical mitoses are reported in Table 1.

RDEB cSCC and cutaneous pseudoepitheliomatous hyperplasia were excised at an earlier age than in the other three groups ($p<0.005$).

Although not achieving statistical significance, lesion location appeared different between the RDEB groups (both cSCC and cutaneous pseudoepitheliomatous hyperplasia) than the other three groups (primary, secondary and OTR kidney cSCC).

Figure 1 illustrates histopathological differences between RDEB cSCC and secondary cSCC; the tumor surrounding connective tissue in RDEB cSCC presents a minor desmoplastic reaction, and a remarkably lower lymphocytes infiltration. The median number of atypical mitoses per field was not different between RDEB and the other cSCC groups, while the number of mitoses

was significantly higher in secondary compared to primary cSCC (p = 0.03). Only one patient from the cSCC-RDEB group had cancer recurrence four years later than the first cSCC (the cSCC analyzed in this study); after 15 months, the patient died due to lymph nodes and visceral metastases. This patient, at IHC evaluation, had a lower peritumoral cellular infiltrate than other patients.

Immunohistochemical results of the tumoral infiltrate

A significant reduction of CD3+,CD4+,CD68+ between the cSCC-RDEB compared to primary cSCC and CD3+, CD4+, CD8+, CD20+ was observed in cSCC-RDEB compared to secondary cSCC. On the contrary there was no difference in CD3+, CD8+, CD20+, CD68+ expression when comparing cSCC-RDEB to kidney OTR cSCC. (Figure 2).

CD3 expression was 6.76 ± 1.46 (cells/mm^2) in the group of cSCC RDEB patients; 19.38 ± 4.40 (cells/mm^2) in the primitive cSCC group; 21.82 ± 4.90 (cells/mm^2) in the secondary cSCC group; 11.4±1.25 (cells/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and 9.94 ± 1.06 (cells/mm^2) in the kidney OTR cSCC group. The difference was statistically significant between the first and second group (p value 0.0262); between the first and third group (p value 0.0187); between the first and fourth group (p value 0.0428) and between the third and fifth group (p value 0.045) (Figure 2, Figure 3).

CD4 expression was 1.86 ± 0.82 (cells/mm^2) in the group of cSCC RDEB patients; 10.50 ± 3.41 (cells/mm^2) in the primitive cSCC group; 12.30 ± 2.78 (cells/mm^2) in the secondary cSCC group; 6.62±2.02 (cells/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and 8.36 ± 0.70 (cells/mm^2) in the kidney OTR cSCC group. The difference was statistically significant between the first and second group (p value 0.0392), between the first and third group (p value 0.0071) and between the first and fifth group (p value 0.0003) (Figure 2, Figure 3, Supplemental Table 1).

CD8 expression was 5.82 ±1.51 (cells/mm^2) in the group of cSCC RDEB patients; 10.42 ± 2.78 (cells/mm^2) in the primitive cSCC group; 11.84 ± 1.78 (cells/mm^2) in the secondary cSCC group; 5.74±1.201 (cells/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and 6.48 ± 0.95 (cells/mm^2) in the kidney OTR cSCC group. The difference was statistically significant between the first and third group (p value 0.0165), between the third and fourth group (p value 0.0086) and between the third and fifth (p value 0.0104) (Figure 2, Figure 4).

CD20 expression was 6.68 ± 2.74 (cells/mm^2) in the group of cSCC RDEB patients; 20.02 ± 5.69 (cells/mm^2) in the primitive cSCC group; 19.12 ± 3.81 (cells/mm^2) in the secondary cSCC group; 7.34±2.50 (cells/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and 7.82 ±

1.08 (cells/mm^2) in the kidney OTR cSCC group. The difference was statistically significant between the first and third group (p value 0.0294), between the third and fourth group (p value 0.0325) and between the third and fifth group (p value 0.0215) (Figure 2, Figure 4).

CD68 expression was 8.10 ± 1.18 (cells/mm^2) in the group of cSCC RDEB patients; 14.04 ± 1.52 (cells/mm^2) in the primitive cSCC group; 9.96 ± 1.82 (cells/mm^2) in the secondary cSCC group; 10.74 ±1.15 (cells/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and 6.44 ± 0.47 (cells/mm^2) in the kidney OTR cSCC group.

As to RDEB cSCC, the difference was statistically significant only with primary cSCC in immunocompetent individuals (p value 0.0153). In addition, there was a statistically significant difference between the second and fifth group (p value 0.0005) and between the fourth and fifth group (p value 0.0086) (Figure 2, Figure 5).

EGFR expression was 2.20 ± 0.83 (signals/mm^2) in the group of cSCC RDEB patients; 2.60 ± 0.55 (signals/mm^2) in the primitive cSCC group; 2.67 ± 0.51 (signals/mm^2) in the secondary cSCC group; 2.40 ±0.24 (signals/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and in the kidney OTR cSCC group 2.80 ± 0.45.The differences were not statistically significant : between the first and fifth group (p value 0.4048), between the fourth and fifth (p value 0.5238) and among the other groups (p value >0.99) (Figure 2, Figure 5).

Foxp3 expression was 1.76 ± 0.51 (cells/mm^2) in the group of cSCC RDEB patients; 3.54 ± 1.65 (cells/mm^2) in the primitive cSCC group; 3.40 ± 0.97 (cells/mm^2) in the secondary cSCC group; 1.30 ± 0.68 (cells/mm^2) in the cutaneous pseudoepitheliomatous hyperplasia RDEB group and 5.54 ± 1.22 (cells/mm^2) in the kidney OTR cSCC group. The difference was statistically significant between the first and fifth group (p value 0.021) and between the fourth and fifth group (p value 0.0165) (Figure 2, Figure 6).

In our samples we found for PDL1 complete negativity or a focal positivity approximately of 1-2% of neoplastic cells, which did not allow us to carry out an adequate count and statistical analysis. Subsequently we colored some samples with the approved kit for the diagnosis of lung carcinoma to check the genuineness of our preparations. Also in this second test we obtained the same result as the experimental kit.

DISCUSSION:

Cutaneous squamous cell carcinoma (cSCC) is a complication of inherited epidermolysis bullosa, which occurs especially in the recessive dystrophic form, resulting more aggressive, as well as more frequent than basal cell carcinoma and melanoma. Cancer has an early onset in RDEB patients, and

the cumulative risk rises with age [6]. The sites most commonly involved are the limbs, both legs (54.7%) and arms (30.8%), and less commonly, the mucosa (8.6%)[5].

In RDEB patients, cSCC are generally well-differentiated tumors. This was confirmed by Montaudiè et al., who reported a well-differentiated tumor rate of 73.9% versus 18.2% and 7.9% for moderately and poorly differentiated tumors, respectively[5]. Nevertheless, these tumors have a very aggressive biological behavior and, despite radical surgery, are fatal within 5 years in about 80% of patients due to locoregional and visceral metastases[6].

In our experience, cases of cSCC in RDEB patients show an earlier onset, as confirmed in the literature[5], where the mean age is about 36 years of age. This is attributable to the particular conditions of patients with RDEB, whose biochemical composition of the skin and immune response to irritant and carcinogenic stimuli are both anomalous.[13] In our study, in patients with kidney OTR the age of cSCC development has an intermediate value between the RDEB groups and other two groups. Additionally, the sites involved in RDEB patients are different compared with those in the other three groups of cSCC patients. The highest incidence is on the limbs, consistent with the literature where the reported frequency is 85.5 %[5].

The triggering factor seems to be the greater risk of trauma in these zones (correlated to a local increase of transforming growth factor β[14]).

The anti-tumoral defense mechanism of immune cells was first described in 1863 by Virchow.[15] The immune cells form an infiltrate in the tumoral and peritumoral sites. Various studies have been conducted to assess the tumoral infiltrate in actinic keratosis (AK), in *in situ* SCC and in invasive SCC as compared to healthy skin.[16] A progressive increase in CD3, CD4 and CD8 was found from healthy skin to AK and *in situ* SCC, reaching the highest values in invasive SCC in the tumoral and peritumoral infiltrate.

The infiltrate was also studied in a recent work focused on overall survival and disease-free survival. The results demonstrated that tumor-infiltrating lymphocytes (TILs) with a rich content of CD3 and CD8 increase survival and reduce the incidence of recurrence, while Foxp3 is associated only with a better survival.[17]

Finally, in cSCC arising in patients undergoing immunosuppressive therapy after kidney transplant, lower values of CD4, CD8 and CD20 were found at the margins of the lesion.[18]

EGFR (Epithelial Growth Factor Receptor) is a transmembrane receptor of the Tyrosine Kinase family that binds EGF (Epithelial Growth Factor) and the transforming growth factor α.[19-23]

The relation between EGFR and cSCC in patients with epidermolysis bullosa has been little considered in the literature. Kivisaarii et al[24]. demonstrated that patients affected by cSCC on RDEB had a greater activation of metalloproteinase-7 (MMP-7) than the controls; after interacting

with EGF bound to heparin (Heparin Binding EGF, Hb-EGF) it was transformed into soluble form, activating the EGF receptor. A greater presence of EGF stimulates a higher number of EGFR; therefore it is reasonable that in subjects affected by RDEB the increased MMP-7 is correlated to EGFR activation and hence tumorigenesis.[24] In our experience the EGFr expression was markedly positive in all five groups, with no significant differences between them.

The positivity of EGFR expression in cSCC in RDEB patients confirms the validity of cetuximab therapy in metastatic cSCC administered in a RDEB patient.[25]

In our experience the presence of CD3 lymphocytes was lower in RDEB patients, both in the cSCC group and cutaneous pseudoepitheliomatous hyperplasia group compared to the primitive and secondary cSCC groups. The kidney OTR cSCC group presented an intermediate lymphocytes CD3 cellular density between that of cSCC in RDEB patients and in RDEB cutaneous pseudoepitheliomatous hyperplasia. This indicates a worse deficit in the cSCC-RDEB group of the cell-type immune response in the impaired local peripheral immunity group than the immunosuppressive therapy cSCC group. The finding is supported by the evidence that patients with RDEB also have a central deficiency of this type of immunity.[26]

However, there are no studies in literature describing the perilesional immune infiltrate in subjects affected by RDEB. Recent studies [16,17] have confirmed the increased peritumoral infiltrate of CD3 lymphocytes in cSCC of patients without RDEB.

Our study shows that in cSCC RDEB patients there is a lower expression of CD4 T-helper lymphocytes than in the other groups. Cutaneous pseudoepitheliomatous hyperplasia in subjects affected by RDEB presented an intermediate lymphocytes CD4 cellular density between that of cSCC RDEB patients and of kidney OTR cSCC patients. This has not been previously reported in the literature on cSCC patients affected by RDEB, whereas the increased infiltrate of peritumoral CD4 is known in the other non-RDEB cSCC groups [16] and in the kidney OTR cSCC group.[18]

The deficit of helper lymphocytes has an intrinsic value in quantifying the immune response, featuring a lower antitumoral response capacity as well as a reduced capacity to recruit B lymphocytes, Natural Killer lymphocytes and macrophages.

There was no significant difference in Foxp3 TILs between RDEB cSCC and primary and secondary cSCC and cutaneous pseudoepitheliomatous hyperplasia. Instead, there was a significant difference between RDEB patients (both sSCC and cutaneous pseudoepitheliomatous hyperplasia) and the kidney OTR cSCC group.

CD8 lymphocytes and B lymphocytes (CD20) were significant reduced in the RDEB groups (both cSCC and cutaneous pseudoepitheliomatous hyperplasia) as compared to the secondary but not the primitive form. On the contrary, the kidney OTR cSCC presented a similar CD8 and CD20 infiltrate

to that in the RDEB groups. Similar reports showing a low peritumoral CD8 and CD20 in primitive cSCC [16,17] and low TILs CD4, CD8, CD20 are present in kidney OTR cSCC in literature.[18] On the contrary, there are no studies of the infiltrate in cutaneous pseudoepitheliomatous hyperplasia or cSCC of RDEB patients.

These data about primary cSCC need more investigation because there is a large sample dispersion. According to our data, a barely detectable level of collagen VII was described in literature in the T-cell zone compared to the B-cell zone in RDEB mice spleen.[11] The presence of monocytic-macrophagic cells (CD68) was markedly lower in the group of cSCC in RDEB patients and the kidney OTR cSCC group than in the other three groups.

In many tumors, a reduced peritumoral inflammatory infiltrate has a negative impact on patients outcome, confirming our findings on the CD68 infiltrate. Foll et al.[27] had described an increased immune proteins expression in RDEB-cSCC compared to low-risk cSCC, analysing proteins expression and bacteria infiltration, while no data were indicated on cell infiltrates. Our aim was to analyse CD68 and other immune cell markers to better understand humoral and resident cell infiltration.

A very recent study written by Oh et al.[28], investigated PDL1 expression in 29 cSCC with different grading (well, moderately and poorly differentiated). Accordingly to our results, PDL1 immunoreactivity was detected in a small number of cSCCs and the authors found no significant difference in the proportion of PDL1 immunoreactivity according to the degree of cSCC differentiation.

The data on grading, size, histological variants, and number of atypical mitoses were not statistically significant. This result is due to poor statistical power owing to the small sample size.

On the basis of our findings and literature reports, we hypothesize that the onset of SCC in patients affected by RDEB may be correlated to a reduced immune defence, as an expression of deficient cell-mediated immunity.[9,26,29] Nevertheless, the reduced peritumoral infiltrate may be the expression of a local deficit. This has recently been analyzed[11], explaining the role of collagen VII as an extracellular matrix immune protein that binds the protein cochlin in the lumen of lymphoid organs.[30] The lack of collagen VII is connected with low levels of macrophages and neutrophil-stimulating cochlin during bacterial infections. Therefore, patients affected by RDEB are more sensitive to the commensal bacteria that often colonize skin wounds and nasal cavities.

The described bacterial colonization is a true “inflammatory burden” in which the bacterial flagellin binds Toll-Like Receptor 5, causing overexpression of the High Mobility Group Box 1 protein, that contributes to the development of cSCC.[31]

It can be hypothesized that a condition of immune tolerance toward the tumor develops in epidermolysis bullosa patients, as a consequence of these deficits, that favors tumor growth. Further studies on larger patients samples are needed to better analyse the aggressive behaviour of cSCC in RDEB patients.

Figure legend:

Figure 1: Hematoxylin eosin of cutaneous squamous cell carcinoma (cSCC) in a RDEB patient (A) and in a patient with a secondary cSCC (B). Both tumors are well-differentiated (G1). Original magnification x 10 (A,B).

Figure 2: Significant reduction of CD3+, CD4+, CD8+, CD20+, CD68+, FOXp3 in cSCC-RDEB compared to controls.

CD3: The difference was statistically significant between the first and second group (p value 0.0262); between the first and third group (p value 0.0187); between the first and fourth group (p value 0.0428) and between the third and fifth group (p value 0.045).

CD4: The difference was statistically significant between the first and second group (p value 0.0392), between the first and third group (p value 0.0071) and between the first and fifth group (p value 0.0003).

CD8: The difference was statistically significant between the first and third group (p value 0.0165), between the third and fourth group (p value 0.0086) and between the third and fifth (p value 0.0104).

CD20: The difference was statistically significant between the first and third group (p value 0.0294), between the third and fourth group (p value 0.0325) and between the third and fifth group (p value 0.0215).

CD68: The difference was statistically significant only between the first and second group (p value 0.0153), between the second and fifth group (p value 0.0005) and between the fourth and fifth group (p value 0.0086).

Foxp3: The difference was statistically significant between the first and fifth group (p value 0.021) and between the fourth and fifth group (p value 0.0165).

EGFr: No significant EGFR expression in cSCC-RDEB compared with controls. The differences were not statistically significant: between the first and fifth group (p value 0.4048), between the fourth and fifth (p value 0.5238) and among the other groups (p value >0.99).

Significance:*(p value <0.05),**(<0.01),***(<0.001).

Figure 3: CD3 immunohistochemical reaction (IHC) of cSCC in a RDEB patient (A) and in a patient with secondary cSCC (B). The CD3+ T lymphocytes infiltration was extended from the connective tissue surrounding the carcinoma to the epidermal basal and suprabasal layers and appear less numerous in the RDEB patient (C) as compared with the patient with secondary cSCC (D).

Original magnification x4 (A,B,C,D).

Figure 4: CD8 IHC of cSCC in a RDEB patient (A) and in a patient with secondary cSCC (B). The lymphocytes T infiltration is higher in secondary cSCC than in cSCC-RDEB.
CD20 IHC of cSCC in a RDEB patient (C) and in a patient with secondary cSCC (D). The B lymphocytes infiltration is remarkable in dermis as cellular nodules. Original magnification x4 (A,C,D) and x20(B).

Figure 5: CD68 IHC of cSCC in a RDEB patient (A) and in a patient with secondary cSCC (B). The CD68 positive cells have cytoplasmic extensions; Langerhans cells are also present.
EGFR IHC of cSCC in a RDEB patient (C) and in a patient with secondary cSCC (D). Original magnification x10 (A) x4 (B,C) and x20 (D).

Figure 6: Foxp3 IHC in a RDEB patient (A) and in kidney OTR cSCC (B). The Foxp3 TILS is remarkable in the kidney OTR cSCC peritumoral dermis. Original magnification x4 (A,B).

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