Journal of
Otorhinolaryngology, Hearing and Balance Medicine

MDPI

Article

Occurrence of Human Defensins and S100 Proteins in Head and Neck Basal Cell Carcinoma (BCC) Entities: hBD3 and S100A4 as Potential Biomarkers to Evaluate Successful Surgical Therapy

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Abstract: Background: The goal of this study is the identification of potential marker molecules for characterizing different basal cell carcinoma entities, to help improve clinical decisions for surgical resection therapy. Methods: Three different entities, sclerodermiform, solid and superficial basal cell carcinomas, were subjected to immunohistochemical microscopy and histomorphometric analyses for human α- (DEFA1/3; DEFA4) and β-defensins (hBD1/2/3) and special S100 proteins (S100A4/7/8/9). Thirty specimens of the three entities were evaluated. Analyses were performed by comparing tissue and cellular localization and staining intensities of tumorous with non-tumorous areas. Staining intensities were semiquantitatively examined by using an RGB-based model. Results: Human defensins are present in all three entities of basal cell carcinomas. They all show cytoplasmic immunostaining in cells of the epithelium, stroma and tumor. Notably, human β-defensin3 is accumulated in the cell nuclei of sclerodermiform and superficial basal cell carcinomas. S100A4 and A7 are undetectable in tumor regions. However, S100A4 occurs in cancer-associated stroma cells with nuclear staining in superficial basal cell carcinomas. Conclusion: Two candidates, namely hBD3 and S100A4, might be used as potential clinical tools for evaluating successful surgical resection therapy to avoid aesthetic and functional facial deformation.

Keywords: basal cell carcinoma; molecular pathology; human defensins; S100 proteins; biomarker; surgical resection treatment

Citation: Dröge, E.; Probstmeier, R.; Wenghoefer, M.; Winter, J. Occurrence of Human Defensins and S100 Proteins in Head and Neck Basal Cell Carcinoma (BCC) Entities: hBD3 and S100A4 as Potential Biomarkers to Evaluate Successful Surgical Therapy. *J. Otorhinolaryngol. Hear. Balance Med.* **2023**, *4*, 1. https://doi.org/10.3390/ohbm4010001

Academic Editor: Norihiko Narita

Received: 28 January 2023
Revised: 17 February 2023
Accepted: 20 February 2023
Published: 22 February 2023

1. Introduction

Basal cell carcinomas (BCCs) are well-recognized as the most common malignancy in humans, although cancer registries do not frequently collect data on this skin cancer [1,2]. Nevertheless, the American Cancer Society reported in a survey in 2010 a total of two million people treated with non-melanoma skin cancer (NMSC) in 2006, with an estimated risk of 3.5 million for NMSCs in this period [3,4]. The estimated lifetime risk to develop skin cancer in the United States is 20%. Most of this risk is associated with NMSCs [5].

BCCs are divided by their clinical features into several subtypes: nodular, superficial and morpheaform. The most frequent clinical subtype, nodular BCC (up to 80% of all BCCs), is found in 90% of the patients in the head and neck region, predominantly on the cheeks, forehead, nasolabial fold or around the eyes [1,6,7]. BCCs are slow-growing lesions of epidermal basaloid cells. Although this tumor entity only very rarely metastasizes, nevertheless BCCs can cause severe tissue destructions because of local invasion [8]. The main risk factor is exposure to ultraviolet radiation, which explains why BCCs mainly occur in sun-exposed areas [9]. The molecular explanation for this clinical observation is a genetic alteration/mutation in the so-called "UV signature" in the genome [10].

Based upon histopathological features, nodular and superficial BCCs are associated with an indolent growth type, whereas morpheaform, infiltrative, micronodular and basosquamous BCCs are correlated with an aggressive growth type, causing extensive local tissue destruction and recurrence [11,12].

Although a number of treatment options do exist—such as topical imiquimod application [13], photodynamic therapy [14], cryotherapy [15] or radiation [16]—the gold standard is the complete removal of the tumor by Mohs micrographically controlled surgery (MMCS) [1,17].

Bearing in mind that most of the BCCs occur in the head and neck region, it appears to be of tremendous importance that the resections are performed under the avoidance of unnecessary sacrifice of healthy tissue, on one hand, but also under the avoidance of re-resections after incomplete primary resections, on the other hand [18].

This dilemma of the plastic surgeon leads to the question of whether any additional markers do exist. Such tools could help to reduce the safety distance in resection margins and to avoid repeated re-resections [6,7,18].

A number of recent studies suggest that antimicrobial proteins (AMPs), like human defensins and members of the S100 protein family, seem to play a key role in carcinogenesis and tumor progression in head and neck cancers [19–31], e.g., in oral squamous cell carcinoma (OSCC) [20–22,25,28,30,31] or tumors of salivary glands [19,23,26–29]. The gene expression of human β-defensins has also already been described in BCCs, but on transcript level only [32]. However, so far no studies on cellular distribution and the occurrence of human defensins and specific S100 proteins in BCCs, nor any impacts on clinical application have been reported recently.

Human defensins belong to the large group of AMPs which are involved in host defense [33]. Besides this important function in innate immunity, it has become apparent over the last years that defensins play various relevant roles in further cellular processes, such as inflammation, wound healing, proliferation and differentiation [34]. There are two subgroups of human defensins, called α- (DEFAs) and β-defensins (hBDs). DEFAs are primarily located in phagocytes, while hBDs are present in epithelial tissues [28,33–36]. However, these AMPs have also been found in benign, precancerous and tumor tissues [28,32]. Hence, tumor-related functions of these peptides were suggested [28,37,38], such as a tumor suppressor for hBD1 [19,37,38] or as ligands of epidermal growth factor receptor (EGFR) for DEFAs and hBDs based upon structure similarities to EGF [39,40].

The S100 protein family consists of at least 20 members, which are characterized by their calcium-binding properties and typical EF-hand-type motifs [41]. Despite their structural similarity, these proteins participate in various different cellular processes such as, e.g., host defense, inflammation, proliferation and malignant transformation [42]. Thus, S100 proteins play an important role in the axis of infection–inflammation–tumorigenesis [41–48]. As mentioned above, S100 proteins have already been identified in benign, premalignant and cancerous oral tissues [22,24,28–31,49,50]. The main focus in these studies was put on S100A4 (metastasin), S100A7 (psoriasin) and S100A8/A9 (calprotectin). The latter three show antimicrobial activities [47,51,52], whereas all four S100 proteins can serve as ligands for receptors of advanced glycation end-products (RAGE), which makes them to key players in the formation of an inflammation-based tumor-supportive microenvironment [43].

Research on AMPs, including human defensins and S100 proteins in head and neck cancer, is well-established within our group. Hence, we conducted this study to investigate whether there is a difference of expression and distribution in indolent growth type BCCs in comparison with aggressive growth type BCCs. For this reason, we compared the expression of human β-defensins (hBDs) 1-3, human α-defensins (DEFA) 1-4 and S100A4/A7/A8/A9 in nodular (solid) and superficial BCCs (indolent growth type) with morpheaform, infiltrative, micronodular and basosquamous (sclerodermiform) BCCs (aggressive growth type). As the above mentioned defensins and S100 proteins seem to have diverse functional activities in innate immunity, we hypothesized that their expression

might also play a key role in anti-tumor immune mechanisms involved in tumor cell control after incomplete (R1) resection.

The standard in most clinics is that patients who underwent a primarily R1 resection on a BCC will receive an MMCS re-resection to achieve a complete removal (R0) of the tumor. A common observation during the histopathological investigation of these re-resection specimens is an absence of vital BCC tumor cells, a phenomenon which might be connected to autoimmune processes but is not completely understood at present [1]. For this reason, we investigated whether AMP and S100 proteins might be involved in anti-tumor immune cell control, and thus might serve as a marker to decide whether a re-resection must be performed or left undone in a certain patient. This is of importance in elderly patients with severely restricted health, who are therefore a high risk for surgery [3]. To achieve this aim, we compared the expression of the above described human defensins and S100 proteins in re-resection specimens of primarily R1 resected BCCs containing vital BCC tumor cells with those specimens without traces of BCC tumor cells, assuming that a lack of vital BCC cells is associated with a more sufficient immune response involving the AMPs.

2. Materials and Methods

2.1. The Tissue Sampling

Procedures involving human tissue sampling followed a protocol approved by the ethical board of the University of Bonn (#067/18). All patients had been informed about the study and had signed a letter of informed consent. Non-cancerous and cancerous specimens were taken from the same individuals. Tumor tissue selection was based upon the following parameters (Table 1): sex, age and BCC type.

Table 1. Sex, age and BCC type of patients examined in the present study.

Patient	Sex	Age	BCC Type
1	m	76	sclerodermiform
2	m	84	sclerodermiform
3	m	82	sclerodermiform
4	f	77	sclerodermiform
5	f	81	sclerodermiform
6	m	66	sclerodermiform
7	f	81	sclerodermiform
8	m	69	sclerodermiform
9	f	72	sclerodermiform
10	f	75	sclerodermiform
11	m	76	solid
12	f	84	solid
13	m	81	solid
14	f	89	solid
15	f	62	solid
16	m	73	solid
17	f	71	solid
18	m	71	solid
19	f	53	solid
20	m	65	solid
21	f	85	superficial
22	m	84	superficial

Table 1. *Cont.*

Patient	Sex	Age	BCC Type
23	m	82	superficial
24	f	71	superficial
25	f	80	superficial
26	m	73	superficial
27	f	72	superficial
28	m	71	superficial
29	f	84	superficial
30	m	50	superficial

2.2. Immunohistology

Sequential tumor tissue sections of 2.5 µm thickness were deparaffinized, rehydrated and rinsed with Tris-buffered saline (TBS). Endogenous peroxidase was blocked in a methanol/H_2O_2 solution. Unspecific binding sites were saturated with 1% BSA in TBS. Tissue slices were then incubated in a humid chamber at 4 °C overnight using the following antibodies: rabbit polyclonal anti-hBD1 (Santa Cruz Biotech., Heidelberg, Germany; #sc-20797; 1:25 dilution), rabbit polyclonal anti-hBD2 (Santa Cruz Biotech.; #sc-20798; 1:50 dilution), rabbit polyclonal anti-hBD3 (Santa Cruz Biotech.; sc-30115; 1:50 dilution), rabbit polyclonal anti-human DEFA1/3 (Biotrend, Cologne, Germany; #HDEFA11-S; 1:800 dilution), rabbit polyclonal anti-human DEFA4 (Biotrend; #HDEFA41-S; 1:400 dilution), rabbit monoclonal anti-human S100A4 (Cell Signaling Technology, Leiden, The Netherlands; #13018S; 1:400 dilution), rabbit polyclonal anti-human S100A7 (Santa Cruz Biotech., #sc-67047; 1:100 dilution), rabbit polyclonal anti-human S100A8 (Santa Cruz Biotech.; #sc-20174; 1:50 dilution) and rabbit polyclonal anti-human S100A9 (Santa Cruz Biotech.; #sc-20173; 1:100 dilution). Antigen–antibody binding was visualized using EnVision Detection System Peroxidase/DAB goat anti rabbit from Dako (Hamburg, Germany; #K4002) [19,23,24,26,30,31]. Cell counterstaining was performed with Mayer's haematoxylin. Immunohistochemical staining intensities were evaluated using an RGB-based model [53] in which staining intensities were measured as a percentage of the relative ratio of "R" compared to total "RGB": - = no ("R" = 33–35%), + = weak ("R" = 36–38%), ++ = moderate ("R" = 39–41%) and +++ = strong ("R" = 42–44%). The quantification was carried out with AxioVision SE64 Rel. 4.9 version (Carl Zeiss Microscopy, Oberkochen, Germany). Histomorphometric analyses of non- and tumorous tissue sections of sclerodermiform, solid and superficial basal cell carcinoma (n = 10 each) were investigated by evaluating the ratio of positively and negatively immunostained cells within representative tissue areas. Forty cells of each specimen within a representative area were counted. The tissue sections were analyzed independently by eye [19,23,54]. The h-score was calculated using the staining intensities and percentage of positively stained cells [55].

3. Results

One entity of aggressive-type, namely sclerodermiform BCC, and two entities of indolent-type, including solid and superficial BCCs (Table 1), were immunohistologically and histomorphometrically analyzed regarding the presence, cellular distribution and localization of the above described human defensins and S100 proteins (Tables 2 and 3).

HBD1 occurred with moderate immune intensity in the cytosol of the epithelium (100% positive cells) and stroma (50% positive cells) in the non-tumorous area (Figures 1 and 2) and also with the same grade of intensity in the tumor center (TC) and tumor edge (TE) (Figures 1 and 2) of sclerodermiform BCCs.

Table 2. Tissue-specific immunostaining pattern and histomorphometric analyses of hBD1, hBD2, hBD3, DEFA1/3, DEFA4, S100A4, A7, A8 and A9 in non- and tumorous tissues of sclerodermiform, solid and superficial basal cell carcinoma (n = 10 each). The percentages of positively stained cells in the cytoplasm of epithelium (E), stroma (S) in non-tumorous, and tumor center (TC) or tumor edge (TE) in tumorous specimens were evaluated for quantification and designated as mean values with standard error means in brackets. Nuclear staining is shown with (n) for hBD3 in sclerodermiform and superficial BCCs and for S100A4 in non-tumorous stromal cells of superficial BCCs.

	Sclerodermiform				**Solid**				**Superficial**			
	Non-Tumorous		**Tumor**		**Non-Tumorous**		**Tumor**		**Non-Tumorous**		**Tumor**	
	Epith.	**Stroma**	**Center**	**Edge**	**Epith.**	**Stroma**	**Center**	**Edge**	**Epith.**	**Stroma**	**Center**	**Edge**
hBD1	98 (4)	49 (5)	99 (3)	100	100	18 (6)	98 (4)	97 (6)	100	22 (4)	11 (3)	99 (4)
hBD2	100	71 (5)	53 (6)	100	99 (3)	31 (5)	100	100	100	31 (3)	22 (4)	97 (6)
hBD3	100	99 (3)	97 (6)	100 n/52 (4)	100	12 (4)	97 (6)	99 (3)	100	98 (4)	23 (8)	97 (3) n/25 (5)
DEFA1/3	97 (6)	48 (6)	99 (3)	100	98 (4)	49 (7)	100	79 (5)	100	99 (3)	12 (4)	97 (6)
DEFA4	100	11 (3)	100	98 (4)	99 (3)	13 (4)	100	97 (6)	100	12 (4)	12 (4)	98 (4)
S100A4	0	68 (6)	0	0	0	53 (6)	0	0	0	51 (6) n/48 (4)	0	0
S100A7	81 (5)	0	0	0	89 (5)	0	0	0	82 (4)	0	0	0
S100A8	88 (4)	0	99 (3)	100	90 (6)	0	8 (6)	100	81 (5)	0	78 (6)	99 (3)
S100A9	100	0	51 (5)	99 (3)	98 (4)	0	99 (3)	100	97 (6)	0	0	0

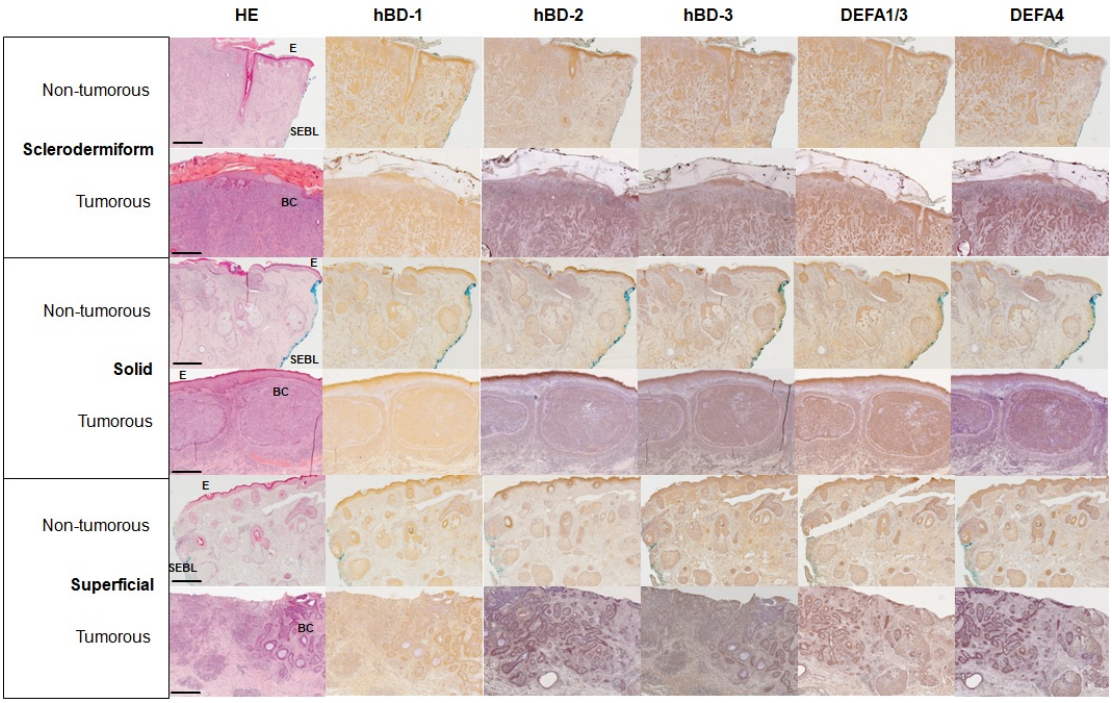

Figure 1. Immunohistological overview of representative sections from non-tumorous and tumorous sclerodermiform, solid, and superficial basal cell carcinomas showing micrographs with HE, hBD1, hBD2, hBD3, DEFA1/3 and DEFA4 staining. The bar represents 500 µm. Primary magnification was 5-fold. Surgery excision borderline (SEBL) is shown in blue staining. Abbr.: E = Epithelium; BC = Basaloid Cells.

Table 3. Tissue-specific immunostaining pattern and histomorphometric analyses of hBD1, hBD2, hBD3, DEFA1/3, DEFA4, S100A4, A7, A8 and A9 in non- and tumorous tissues of sclerodermiform, solid and superficial basal cell carcinoma (n = 10 each). The color intensity and h-scores in the cytoplasm (c) or nuclei (n) of epithelium (E), stroma (S) in non-tumorous, and tumor center (TC) or tumor edge (TE) in tumorous specimens were evaluated for quantification. Staining intensities are depicted as "-" (not detectable), "+" (weak), "++" (moderate) and "+++" (strong).

	Sclerodermiform		**Solid**		**Superficial**	
	Non-Tumorous	**Tumor**	**Non-Tumorous**	**Tumor**	**Non-Tumorous**	**Tumor**
hBD1	E/++/196S/++/98	TC/++/198TE/++/200	E/++/200S/++/36	TC/++/196TE/++/194	E/++/200S/++/44	TC/+/11TE/++/198
hBD2	E/+++/300S/++/142	TC/+/53TE/++/200	E/+++/297S/+/31	TC/++/200TE/++/200	E/+++/300S/+/31	TC/+/20TE/++/194
hBD3	E/++/200S/+/99	TC/+/97TE/+/c/100TE/++/n/104	E/++/200S/+/12	TC/++/194TE/++/198	E/++/200S/++/196	TC/++/46TC/++/c/198TE/++/n/50
DEFA1/3	E/++/194S/+/48	TC/++/198TE/++/200	E/++/196S/+/49	TC/++/200TE/++/158	E/++/200S/+/198	TC/++/24TE/++/194
DEFA4	E/++/200S/+/11	TC/++/200TE/++/196	E/+/99S/+/13	TC/++/200TE/++/194	E/++/200S/+/12	TC/+/11TE/++/196
S100A4	E/-/0S/+/68	TC/-/0TE/-/0	E/-/0S/+/53	TC/-/0TE/-/0	E/-/0S/+/c/51S/+/n/48	TC/-/0TE/-/0
S100A7	E/+/81S/-/0	TC/-/0TE/-/0	E/++/178S/-/0	TC/-/0TE/-/0	E/++/164S/-/0	TC/-/0TE/-/0
S100A8	E/++/176S/-/0	TC/+/198TE/+/100	E/++/180S/-/0	TC/+/88TE/+/100	E/+++/243S/-/0	TC/+/78TE/+/99
S100A9	E/+++/300S/-/0	TC/+/51TE/+/99	E/++/196S/-/0	TC/+/99TE/+/100	E/+++/291S/-/0	TC/-/0TE/-/0

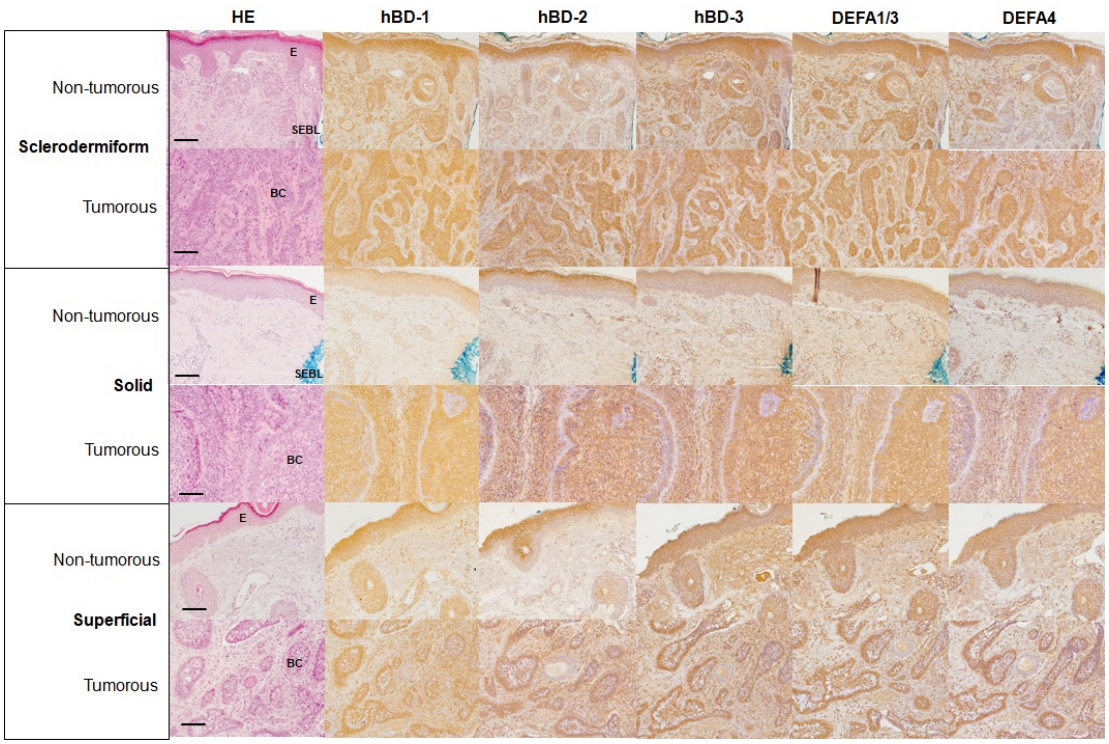

Figure 2. Immunohistological detailed microscopic photographs from non-tumorous and tumorous sclerodermiform, solid, and superficial basal cell carcinomas showing micrographs with HE, hBD1, hBD2, hBD3, DEFA1/3 and DEFA4 staining. The bar represents 100 µm. Primary magnification was 20-fold. Surgery excision borderline (SEBL) is shown in blue staining. Abbr.: E = Epithelium; BC = Basaloid Cells.

Very similar observations could be seen for indolent BCCs (Figures 1 and 2), with the following exceptions: only 20% of hBD1-positive cells were detected in the stroma of non-tumorous areas (Figures 1 and 2), and only 10% of tumor center cells were hBD1-positive in superficial BCCs (Figures 1 and 2). Analyses for hBD2 presence (Figures 1 and 2; Tables 2 and 3) showed only marginal differences compared to hBD1: 1. Immunostaining intensity was generally higher in epithelium layers and to a slightly higher degree of hBD2-positive cell numbers in non-tumorous stroma in all types of BCCs (Figures 1 and 2); 2. In sclerodermiform tumor areas, only 50% of hBD2-positive cells were observed (Figure 2); 20% hBD2-positive cells in superficial tumor regions (Figure 2). In contrast, hBD3 analyses showed a different picture: in sclerodermiform BCCs, 50% of tumor edge cells were hBD3-positive for nuclear localization (Figure 2; Tables 2 and 3) while this AMP exhibited nuclear as well as cytosolic staining in 25% of tumor center cells in superficial BCCs, with nuclear and cytosolic staining remarkably not occurring in the same cells (Figure 2; Tables 2 and 3). The patterns of DEFA1/3s' presence were very similar to the above described hBDs (Figures 1 and 2). However, DEFA1/3 immunostaining intensities were weak in stroma cells of non-tumorous regions in all entities (Figures 1 and 2; Tables 2 and 3). The specific difference for DEFA4 occurrence in BCCs was the low amount of DEFA4-positive cell numbers in stroma of non-tumorous areas within all three entities (Figures 1 and 2; Tables 2 and 3).

In contrast to the above-described defensins, S100A4 could not be detected in epithelial cells of non-tumorous sclerodermiform BCCs, yet was present in the cytosol of 70% of stromal cells with weak intensity, but also completely absent in tumorous areas (Figures 3 and 4; Tables 2 and 3).

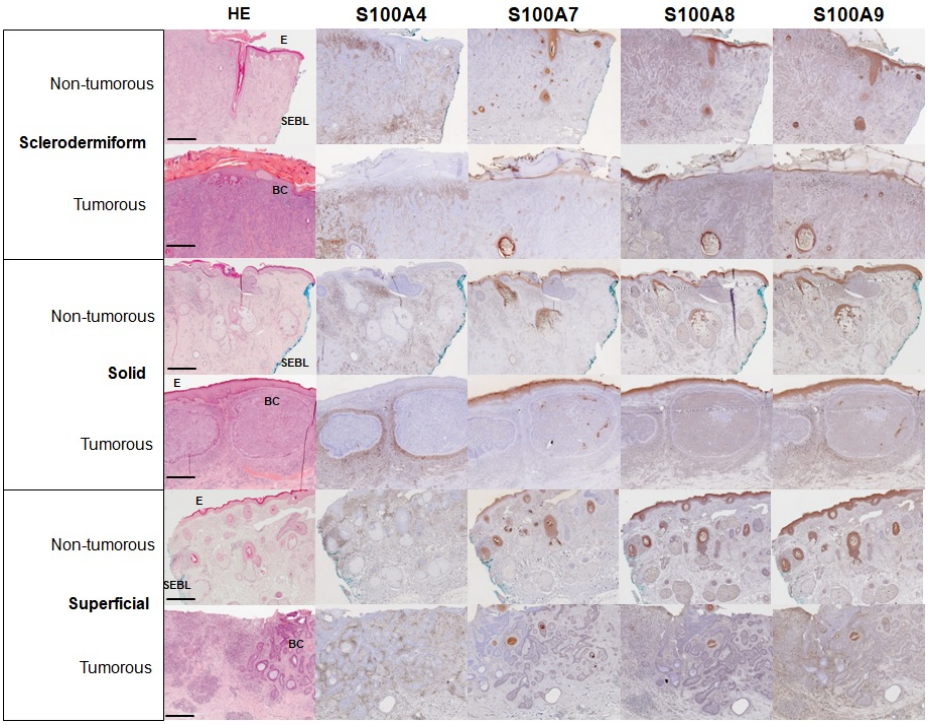

Figure 3. Immunohistological overview of representative sections from non-tumorous and tumorous sclerodermiform, solid, and superficial basal cell carcinomas showing micrographs with HE, S100A4, A7, A8 and A9 staining. The bar represents 500 µm. Primary magnification was 5-fold. Surgery excision borderline (SEBL) is shown in blue staining. Abbr.: E = Epithelium; BC = Basaloid Cells.

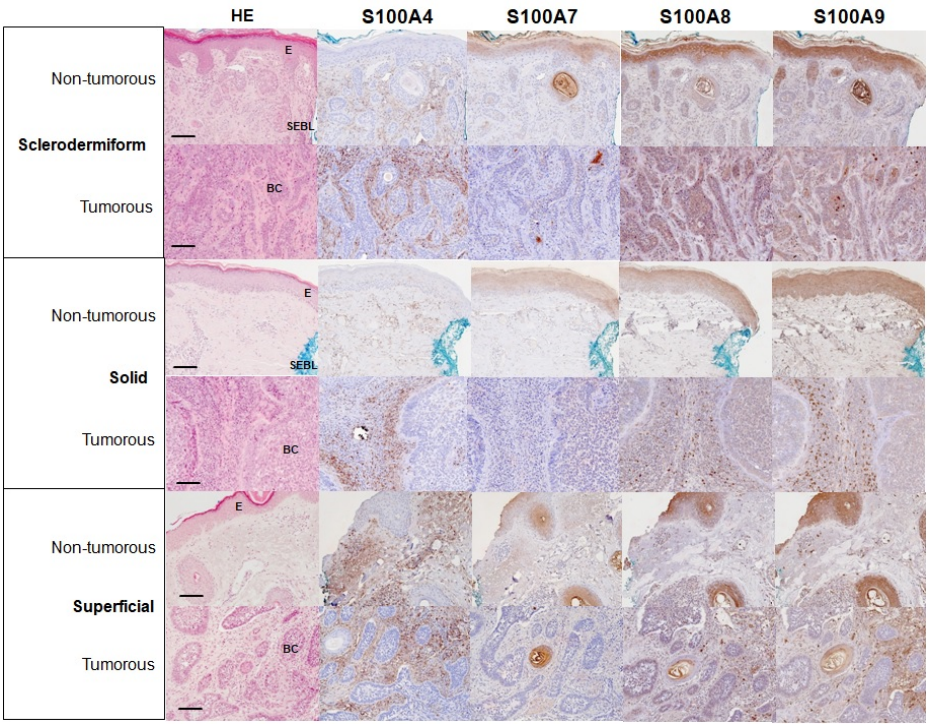

Figure 4. Immunohistological detailed microscopic photographs from non-tumorous and tumorous sclerodermiform, solid, and superficial basal cell carcinomas showing micrographs with HE, S100A4, A7, A8 and A9 staining. The bar represents 100 µm. Primary magnification was 20-fold. Surgery excision borderline (SEBL) is shown in blue staining. Abbr.: E = Epithelium; BC = Basaloid Cells.

Similar results were observed for S100A4 occurrence in non-tumorous and tumorous regions of solid BCCs (Figures 3 and 4; Tables 2 and 3). In the case of superficial BCCs, S100A4 immunohistological patterns were shown to be slightly different: the epithelium and tumorous areas were S100A4-negative, while 50% of non-tumorous stromal cells showed cytosolic and simultaneous nuclear staining (Figures 3 and 4; Tables 2 and 3). S100A7 was also not detectable in tumorous regions of all three BCC entities examined (Figures 3 and 4; Tables 2 and 3). However, S100A7 reactivity was monitored in the cytoplasm of epithelial cells in all three BCCs tested (Figures 3 and 4; Tables 2 and 3). S100A8 presence was not detectable in stroma cells, but with moderate or even high intensity in the cytoplasm of 80–90% of epithelial cells (Figures 3 and 4; Tables 2 and 3). In sclerodermiform BCCs, all cells of the tumor center and edge were S100A8 positive with cytoplasma localization, which also applied, although to a marginally lower extent, for solid BCCs (80% of cells) and superficial (90%) BCCs (Figures 3 and 4; Tables 2 and 3). S100A9 showed the following patterns: non-tumorous stromal cells of all three BCCs were immuno-negative for this protein, while the epithelium exhibited moderate to strong reactivity in the cytoplasm of all cells. S100A9 was detectable in sclerodermiform BCCs with weak intensity in 50% of cells of the tumor center and 100% in the tumor edge, whereas all cells in solid BCC tumor cells were S100A9 positive, also with weak immunostaining intensity. In contrast, no S100A9 could be detected in superficial BCC tumor areas (Figures 3 and 4; Tables 2 and 3).

4. Discussion

As already mentioned above, human defensins and the S100 members S100A4/A7/A8/A9 play a pivotal role in tumorigenesis and malignant transformation, and also under inflammatory conditions [28,31,43,56]. All these different proteins, with the exception, of S100A4 have in common that they share antimicrobial activities [31,33,34]. However, remarkably, they also participate in cellular processes which are favorable to initiate or establish tumors, e.g., as ligands of EGFR (hBDs, DEFAs) [39,40] and RAGE (S100A4/A7/A8/A9) [43–45]. Furthermore, they also function as tumor suppressor proteins (hBD1/hBD2) [20,37,38] or as intracellular interacting partners, which drive epithelial–mesenchymal transition (EMT) (S100A4/A7) [43,46,56]. Hence, they are key players in the inflammation–tumorigenesis axis. In addition, these proteins have also already been described as useful tools for characterizing various lesions of different grades of malignancy in a number of tumor entities [19–26,28–32,37,38,40,50]. Therefore, this study has been performed to find out whether these molecules might serve as biomarkers for characterizing different BCC entities. This might help in improving clinical decisions for resection therapy. HBD1-3 has been detected in all three different entities of BCCs examined. It is present in the cytoplasm of epithelial, stromal and tumor cells. In addition, hBD3 also occurs in the nuclei of sclerodermiform and superficial BCCs. This observation might lead to the hypothesis that hBD3 has a yet unknown function specific for these two BCC entities. S100A4 and A7 are not detectable in tumor regions. Nevertheless, S100A4 shows a specific immunostaining property in cancer-associated stroma cells with nuclear occurrence in non-tumorous areas of superficial BCCs. Thus, these proteins might participate in tumor suppressing functions. S100A8 and A9 show similarities in expression profiles throughout sclerodermiform and solid BCCs, but differ in superficials. Hence, these proteins could be involved in entity-specific yet unknown functions.

Human defensins have originally been discovered to be antimicrobial peptides. However, a huge number of studies indicate that these AMPs are involved in a wide variety of different cellular processes, including the initiation and progression of skin disorders and neoplasms [57]. Transcript levels of hBD1 are reduced in BCCs compared to those of healthy skin [32]. Additionally, the expression level of this AMP corresponds to the degree of malignancy with the lowest concentration found in the most malignant lesions in skin [58]. This observation has also been found in different stages of malignancies in various oral tissues [20,49]. In the present study, the examined BCC entities have also shown different staining intensities for hBD1: while sclerodermiform and solid BCCs exhibit the

same immunostaining profile, the number of hBD1-positive cells in the tumor center as well as the intensity is decreased in superficial BCCs. Although hBD2 expression is reduced in oral squamous cell carcinoma [59], it is enhanced in BCCs [32]. Our results show the highest expression for hBD2 in solid BCCs compared to the other two entities. This may support the assumption that hBD2 might function as a putative tumor suppressor, since solid BCC is the less malignant entity [10,17]. In contrast, hBD2 is more highly expressed in non-tumorous stroma areas of sclerodermiform BCCs, the most malignant of the three forms, compared to the other two entities. The overexpression of hBD3 has been shown in oral squamous cell carcinomas [21] and oral carcinoma in situ lesions [60]. Human BD3 is known to function as a chemoattractant for tumor-associated macrophages which are involved in tumor initiation and progression [60]. Human BD3 shows a different expression profile compared to the other hBDs: while hBD1 and hBD2 are located exclusively in the cytoplasm of tumor, epithelial and stromal cells, hBD3 appears at least partly in the nucleus of cells in the tumor edge in sclerodermiform BCCs and in the tumor center of superficial BCCs. Nuclear accumulations of hBDs have been reported for hBD1 in cells of salivary gland tumors [19,23] and in keratinocytes of burned skin [61]. HNPs or α-defensins (DEFAs) have been used as molecular tools to characterize various oral benign, premalignant and cancerous lesions and salivary gland tumors [26,49,50]. Whereas DEFA1/3 is up-regulated in benign oral irritation fibromas, DEFA4 expression is unaltered [50]. Transcript levels of DEFA1/3 in premalignant oral leukoplakias are unaltered, while DEFA4 is overexpressed [49]. Malignant salivary gland tumor entities show enhanced levels of DEFA1/3 and also DEFA4. In benign pleomorphic adenomas, DEFA1/3 is only slightly up-regulated but DEFA4 even decreased. In addition, both DEFAs are absent in pleomorphic adenomas, although present in healthy salivary gland cells. However, malignant cystadenolymphomas exhibit DEFA1/3 presence in cells of the tumor centers and edge, whereas DEFA4 only occurs in cells of the tumor edge [26]. In BCCs, both DEFAs show similar expression patterns in all three entities. However, DEFA4 is far less present in stroma cells.

Intracellular S100A4 is involved in various cellular processes such as cell migration, apoptosis and stemness maintenance. If present in the extracellular matrix, S100A4 participates in pro-inflammatory and metastasis-promoting processes, e.g., cell motility and epithelial–mesenchymal transition (EMT). It is able to bind to RAGE or EGFR, hence affecting immune modulation and cell growth. Therefore, this protein can link innate immunity with tumorigenesis [43], and might be a relevant player in BCC pathogenesis. S100A4 has been detected in different dermal tissue structures such as healthy skin, nevi and melanomas, with no significant differential expression in the two latter [62]. However, it has also been described that no S100A4 is present in epidermal tumors [63]. Our results support these results, since no positive S100A4 immunoreactivities have been found in BCCs. Nevertheless, cancer-associated stromal cells show S100A4 occurrence in all three BCC entities. Thus, this protein might serve as a potential clinical marker for evaluating successful resectional therapy. S100A7, also designated as "psoriasin", was first isolated from psoriatic skin lesions as an antimicrobial protein [45,51,64,65]. It acts as an alarmin, chemoattractant and an amplifier of inflammation. Additionally, it affects proliferation and establishes a tumor-tolerogenic microenvironment [43,66]. S100A7 has been identified in various oral lesions. Its expression level correlates with the grade of lesional malignancies [22,24,29,50]. S100A7 expression has also been intensively studied in human skin. The results are similar to those in oral tissues, with the exception that S100A7 has been shown to be down-regulated in early steps of dermal tumor development, with the highest level in preinvasive SCCs but the lowest in invasive SCCs. Although transcript levels of S100A7 are enhanced in BCCs [54,66,67], notably S100A7 protein is absent in various immunohistological studies, including ours [67–69]. S100A8 (calgranulin A) is associated with inflammatory diseases and cancer. Its expression is induced inter alia by ultraviolet radiation. S100A8 is a modulator in inflammation, a natural ligand for RAGE and shows antimicrobial activity. S100A8 has been detected in oral and head and neck squamous cell carcinoma and various psoriatic lesions. Furthermore, calgranulin A is able to form a

heterocomplex with S100A9 [43–45]. S100A9 (calgranulin B) has an impact on immune cell migration, and also functions as an AMP [43,52]. It is often co-expressed with calgranulin A, forming a heterocomplex designated calprotectin. Furthermore, S100A8/A9 is involved in establishing a tumor-supportive microenvironment [70]. S100A9 is expressed in various oral and dermal lesions, but is absent in BCCs [24,30,31,50,69]. Our results indicate independent expression profiles for S100A8 and A9 in BCCs. This observation has also been found in dermal keratinocytes and airway tissues [69,71]. Both proteins are present in sclerodermiform, and solid BCCs S100A9 is absent in superficial BCCs, whereas, in contrast, S100A8 has been identified in this tumor entity, yet with weak intensity. Therefore, these proteins could be used as a marker to differentiate between the three entities.

In summary, human defensins have been identified in three different entities of BCCs. They all show cytoplasmic immunostaining in epithelial, stromal and tumor cells. In addition, hBD3 is accumulated in the cell nuclei of sclerodermiform and superficial BCCs. S100A4 and A7 are absent in tumor regions. Nevertheless, S100A4 shows a specific immunostaining in cancer-associated stroma cells, with nuclear occurrence in non-tumorous areas of superficial BCCs. S100A8 and A9 exhibit similar expression profiles throughout sclerodermiform and solid entities, but differ in superficial BCCs.

In conclusion, from the above-mentioned putative biomarker molecules, two candidates, namely hBD3 and S100A4, seem to be useful as potential clinical tools for evaluating successful resection therapy to avoid aesthetic and functional facial deformation. These two biomarkers help to make improved clinical statements of risk estimations for potential recurrence. It is essential to differentiate tumorous from non-tumorous cells to estimate the success of tumor resections. The use of more reliable biomarkers makes it possible to avoid putative re-resections, with all their undesirable consequences.

Author Contributions: M.W. and J.W. have made substantial contributions to the design, conception, methods and drafting of the manuscript. E.D. characterized and verified tissue specimens. E.D., R.P., M.W. and J.W. have examined tissue sections regarding immunohistomorphometry. All authors made substantial contributions to the acquisition, analysis, or interpretation of data, drafted the work or revised it critically for important intellectual content. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The procedures for human tissue sampling followed a protocol approved by the ethical board of the University of Bonn (#067/18).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Supporting data and materials are available on request to the corresponding author due to privacy restrictions.

Acknowledgments: The authors are grateful to D. Lalaouni for excellent technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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