Int J Clin Exp Pathol 2013;6(12):2855-2863
www.ijcep.com /ISSN:1936-2625/IJCEP1308068

Original Article

Claudins 1, 2, 3, 4, 5 and 7 in solar keratosis and squamocellular carcinoma of the skin

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Received August 28, 2013; Accepted October 9, 2013; Epub November 15, 2013; Published December 1, 2013

Abstract: Claudins are tight junction proteins regulating the paracellular permeability of cell layers. We investigated the expression of claudins 1, 2, 3, 4, 5 and 7 in a sample set consisting of a total of 93 cases representing normal skin, actinic keratoses and squamous cell carcinomas of the skin. There were several changes found in claudin expression. Claudin 1 appeared to be progressively decreased in solar keratosis and skin squamous cell carcinomas compared to normal skin while expression of claudin 2 was increased. With claudins 3 and 5 occasional immunoreactivity was found in squamous cell carcinomas. Claudins 4 and 7 were variably expressed in skin neoplasia compared to normal skin. According to the results expression of claudins 1 and 2 change in parallel with the severity of the epidermal preneoplastic and neoplastic lesions thus probably influencing the disturbed epithelial polarity characteristic of these lesions. Claudin 1 under- and claudin 2 overexpression also lead to a leakier epithelial barrier function of the skin with a resulting damage to skin epithelial resistance. Other claudins investigated in this study did not show progressive changes even though occasional overexpression of them was found in skin squamous cell carcinoma.

Keywords: Tight junction, claudins, squamocellular carcinoma, dysplasia, actinic keratosis

Introduction

Tight junction (TJ) serves as an important structure for the relatively impermeable barrier of the skin. TJs are situated at the most apicolateral surface of the cell membrane where they take part in the formation of intercellular junctions of epithelial cells [1]. Their role is to establish diffusion barrier for solutes and to maintain paracellular gate function for regulating the flux of ions and proteins between the epithelial cells [2, 3]. Furthermore, TJs form a fence-like structure to separate lipids and plasma membrane bound proteins to apical and basolateral compartments [1] to maintain normal polarization of the epithelial cell.

Claudins take part in forming an intact and well functioning TJ. Other TJ proteins like occludin and junctional adhesion molecules take part in the structure of TJs but specifically claudins regulate epithelial paracellular permeability. Because there are several types of claudins, 27 in total [4], some of them also having splice variants, claudin expression in the apicolateral membrane may vary considerably which is the reason for the variance in transelectrical and solute permeability in epithelial cell layers. In human kidney, for example, claudins are differentially expressed in different parts of the tubular segments thus regulating the permeability of tubular cells [5]. Mutations of claudins 16 and 19 lead to hypercalciuric hypermagnesemia with nephrocalcinosis [6]. Similarly, claudins are differentially expressed in the gut and claudin 15 knockout animals develop a megaintestine [7].

In carcinogenesis expression of individual claudins may be underexpressed or overexpressed leading to a dysregulation of tight junctional function [8]. Such a disturbance influences the

microenvironment of the cancer cells and disturbs the fence function seen in normal cells [9]. Dysregulation of TJ structures may also influence cellular proliferation and migration of cells since claudins are attached by their PDZ domains to zona occludin proteins 1, 2 and 3 which, on the other hand, attach themselves to the microfilaments, apg2, AP-1 or SAF-B [10-14].

Another aspect where claudins may play some role in cancer spread is epithelial-mesenchymal transition (EMT) [15]. In EMT carcinoma cells lose their epithelial character and become more mesenchymal like. During this process expression of adhesion molecules (such as E-cadherin) is downregulated, and tumor cells increase their expression of mesenchymal markers such as vimentin and smooth muscle actin [16]. There is also a downregulation of claudins, for example, claudin 1 is downregulated by Snail, a transcription factor activated in EMT [17].

Since claudins are present in epithelial, endothelial and mesothelial cells they are expressed in tumors derived from these cells but may show site and type specific characteristics [18]. Claudins can, in a restricted sense, be used in differential diagnosis between tumors of different types. Epithelial tumors can be distinguished from soft tissue tumors since the latter do not express membrane bound claudin expression [18] except for synovial sarcoma [19] and perineurioma [20]. Additionally, claudin 5 is strongly expressed in angiosarcomas and other endothelium derived tumors by which it can be distinguished from other soft tissue tumors [18].

In skin epidermis many claudins are expressed [8]. Claudin 1 and 7 are detected in all layers and also in the epithelium of follicular structures, claudins 3 and 4 in stratum granulosum while claudin 5 is not expressed at all [21]. There are tight junctions in the granulosa cell layer of the epidermis and claudin 1 knockdown mice die of dehydration due to a disturbance in function of the tight junction [22].

In chemical carcinogenesis of skin, several claudins, like claudin 1, 6 and 11 are downregulated [23]. In squamous cell carcinomas of the skin, claudin expression, like for claudins 1 and 4 many times are associated with areas on keratinization [24]. Claudin expression has also been shown in tumors derived from squamous epithelia of the esophagus or tongue [25, 26]. In esophageal squamous cell carcinoma, loss of claudin 4 expression is associated with a higher rate of metastases while claudin 3 is significantly lower in squamous cell carcinomas than adenocarcinomas [25]. In squamous cell carcinomas of the tongue, claudins 1 and 7 are strongly expressed and claudin 7 expression has an influence on survival [26].

Actinic (solar) keratoses have the potential for malignant transformation. They clinically present as erythematous scaly lesion varying in size and shape, and they have many clinical variants. Evidence shows that cumulative exposure to UV-radiation is behind of the development of actinic keratosis and further progression of solar keratosis to squamous cell carcinoma although unambiguous evidence is still to be obtained. UV-induced mutations of tumor suppressor gene p53 appear in both solar keratosis and squamocellular carcinoma of the skin which supports the theory of solar keratosis being an early event in SCC carcinogenesis [27, 28]. The progression of solar keratosis to carcinoma also correlates with the inactivation of another tumor suppressor gene p16, as loss of heterozygosity of the region encoding p16 is less frequent in solar keratosis than in squamocellular carcinoma of the skin [29].

Expression of different claudins has been detected in many cancer types. Although claudins have been vastly studied, the correlation of claudin expression between solar keratosis and squamous cell carcinoma of the skin has not yet been done. The expression of different claudins in solar keratosis and squamocellular carcinoma could be of diagnostic value as a biomarker of cancer or as an indicator of UV induced carcinogenesis in general and such research may also give information on changes in TJ structures during epidermal carcinogenesis.

This paper reports on the results of claudin 1, 2, 3, 4, 5 and 7 expression in normal skin, solar keratosis and in squamocellular carcinomas obtained by immunohistochemical analysis. The changes in these claudins were studied in parallel with the carcinogenesis process of the skin samples.

Materials and methods

Study material

The study material consisted of 93 patient samples (42 solar keratosis, 32 squamocellular carcinomas and as controls, 19 samples of histologically normal skin sections from resection margins) collected from the paraffin block archives stored in the Department of Pathology of Oulu University Hospital during the years 2004 and 2005. The median of patient's age was 71.0 years (range 31-91 years). Solar keratosis samples included 21 cases of mild, 8 cases of moderate and 13 cases of severe dysplasia. Squamocellular carcinoma specimens consisted of 18 samples of histological grade I, 11 samples of grade II and 3 samples of grade III squamocellular carcinomas. Diagnoses were made according to the current WHO classification criteria [30]. The age and gender of the patients were collected from patient records. All samples were fixed in neutral buffered formalin and embedded in paraffin. The study was approved by the Local Ethics Committee of the Osthrobotnian Hospital District.

Immunohistochemistry

Primary antibodies for immunohistochemistry were purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA) and were designed for formalin-fixed paraffin-embedded tissue sections. Primary antibodies were polyclonal rabbit anti-claudin 1 (clone JAY.8), monoclonal mouse anti-claudin 2 (clone 12H12), polyclonal rabbit anti-claudin 3 (clone Z23.JM), monoclonal mouse anti-claudin 4 (clone 3E-2C1), monoclonal mouse anti-claudin 5 (clone 4C3C2) and polyclonal rabbit anti-claudin 7 (clone ZMD.241).

Sections of 5 μm thick were deparaffinised and rehydrated. Sections were first heated in a microwave oven in tris-EDTA for 10 minutes and then incubated with the primary antibody for 60 minutes. The dilution was 1:50 for all anti-claudins and DAKO EnVision kit was used according to the supplier's instructions for the detection of primary antibody. The colour was developed by DAB and the sections were counterstained with haematoxylin and mounted with Pertex (Leica Microsystems, Germany).

Negative controls were handled as previously described but with the primary antibody replaced by serum or PBS. Normal skin present in sections served as a positive internal control since individual claudins decorated non-tumorous skin, adnexal structures and blood vessels.

The intensity and quantity of immunoreactivity was assessed as follows. Criteria of intensity: - no immunostaining present, + weak staining, ++ moderate staining and +++ strong staining. Criteria of staining quantity of the tumorous areas: - expression in 0-5%, + expression in 6-25%, ++ expression in 26-50%, +++ expression in 51-80% and ++++ expression in 81-100% of cells. For statistical analyses, a summary figure obtained by quantitative and intensity of staining was pooled in two categories, weak or no intensity and moderate-to-strong intensity with cases of >6 pluses representing strong and less than six pluses weak expression.

Statistical analysis

Statistical analyses were performed by IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA). The significances between claudin expression and diagnosis and the severity of disease determined using two-sided chi-square test with probability value less than 0.05 considered significant.

Results

Normal skin epidermis showed significantly stronger staining for claudin 1 than actinic keratosis (AK) and squamocellular carcinoma (SCC) together ($p=0.00004$) or separately ($p=0.0012$ for AK and $p<0.0001$ for SCC) (**Figures 1**, **3A** and **3B**). Claudin 1 was more often positive in AK than in SCC ($p=0.019$) (**Figures 1**, **3B** and **3C**). Within the dysplasia group (mild, moderate, strong) or SCC (grade 1, 2 and 3) no statistically significant difference was seen.

On the other hand, there were more cases with claudin 2 positivity in AK and SCC together than in normal skin ($p=0.040$) but this was not true separately for SCC ($p=0.14$) or AK even though the latter had a strong tendency ($p=0.054$) (**Figure 2**). Moderate of strong dysplasia in AK had more often strong immunoreactivity for claudin 2 than mild dysplasia ($p=0.027$). Such differences were not observed between immunoreactivities between SCC of different grades.

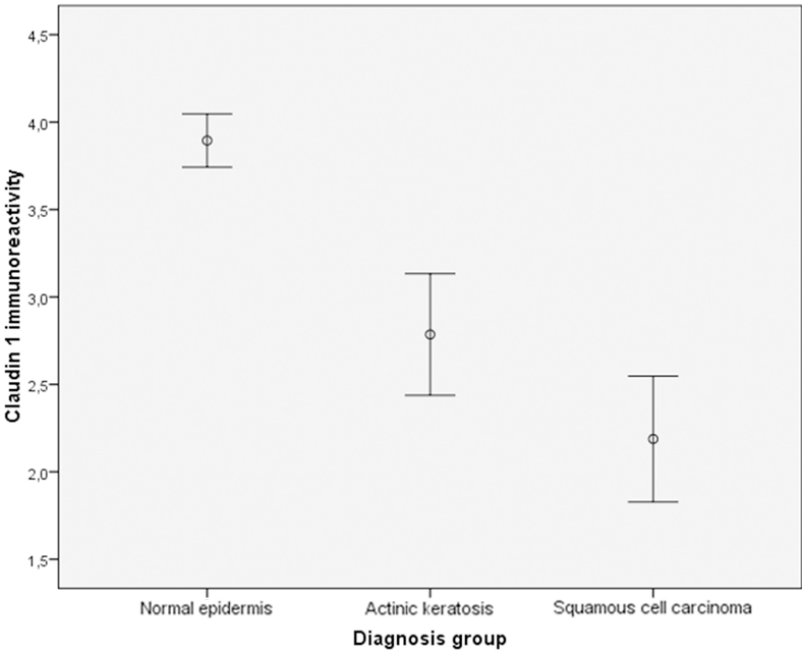

Figure 1. Mean expression of claudin 1 in normal epidermis, actinic keratosis and squamous cell carcinoma. The expression is progressively downregulated.

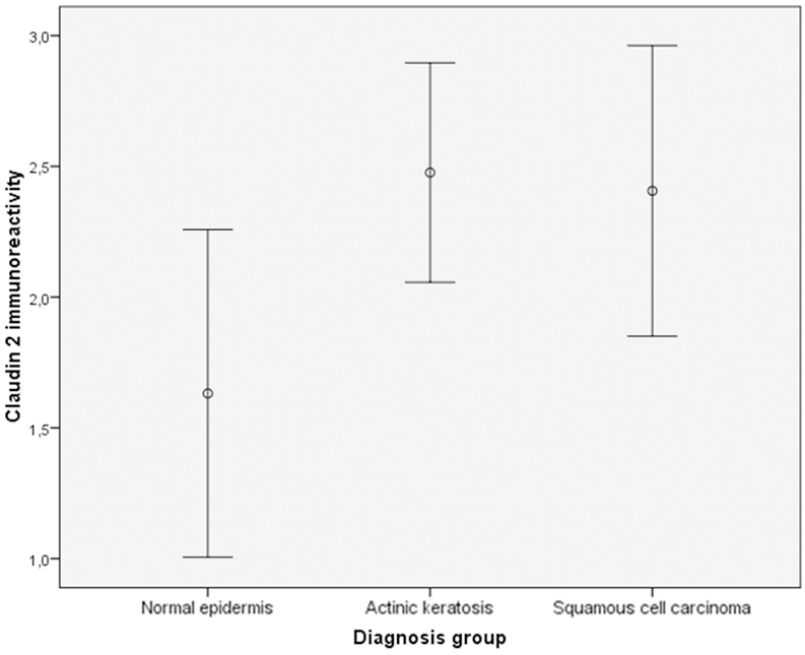

Figure 2. Mean expression of claudin 2 in normal epidermis, actinic keratosis and squamous cell carcinoma. Expression is stronger in actinic keratosis and squamous cell carcinoma than in normal skin.

Claudin 3 was weakly positive only in 2 cases of SCC. Normal skin and AKs were all negative for this antibody. With claudin 5 five cases of SCC showed weak expression. Weak expression was also found in one case of AK and one normal skin.

Strong positivity for claudin 4 was seen in only one case in SCC. Overall positivity was, however, seen in 21 cases of SCC (**Figure 4C**). Weak positivity was seen in 22 AKs and in all cases of normal skin (**Figure 4A** and **4B**). Curiously, AK and SCC together had more cases negative for claudin 4 than normal skin ($p=0.0006$) even though all cases of normal skin had weak positivity.

Two SCCs showed strong claudin 7 expression. Dysplastic skin lesions tended to have more negative cases than SCC ($p=0.057$) but this association was not statistically significant. A significant difference in this respect was, however, observed between SCC and normal skin ($p=0.019$).

Discussion

The main cause of actinic keratosis and a consequent squamous cell carcinoma is exposure to UV radiation. While mutations, like tandem mutations of the p53 gene, is a specific UV irradiation associated genomic change [31], the carcinogenetic cascade, as evaluated by changes in the adhesion molecules like tight junctions, has not been explored. Yet UV irradiation influences the expression of claudins in the skin. It decreases the level of claudin 1 in mouse epidermis and claudin 5 is downregulated in mouse skin lymphatic cells leading to increased oedema due to their leakage [32, 33]. Generally, claudins are also implicated in the epidermal differentiation sug-

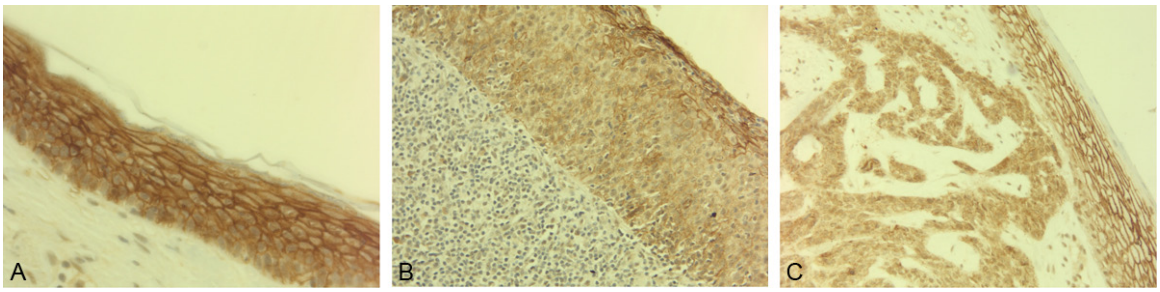

Figure 3. Claudin 1 staining in normal skin. Regular membrane bound expression can be seen in epidermis (A). Claudin 1 expression in severe dysplasia. Membrane bound expression is diminished in the dysplastic keratinocytes (B). In squamous cell carcinoma, only cytoplasmic claudin 1 expression can be detected. The overlying epidermis shows strong membrane bound expression (C).

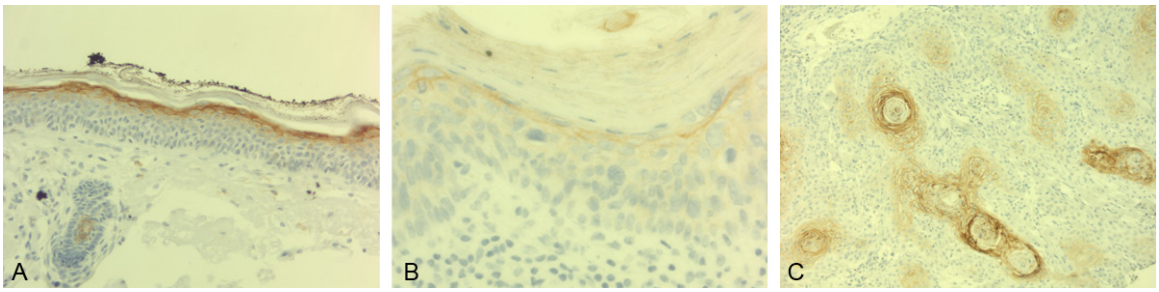

Figure 4. Claudin 4 immunostaining in normal skin. The superficial layers show moderate immunostaining (A). In severe dysplasia, claudin 4 immunostaining appears diminished and the antibody stains only a small rim in the granular/keratin layer. In squamous cell carcinoma of the skin, areas with clear keratinizing differentiation display positivity for claudin 4.

gesting that they may also be involved in neoplasia which is linked to aberrant differentiation [34].

Claudin 1 is detrimental for skin permeability and claudin 1 knockout mice die of dehydration [35]. In our series claudin 1 was strongly expressed in normal epidermis and decreased in solar keratosis and squamocellular carcinomas. This deteriorates the barrier function of the skin with a consequent decrease in the epithelial defence in neoplastic epithelia. Claudin 1 is downregulated by Snail and Slug which both promote EMT in malignant tumors [36]. In line with this, Snail and Slug knockdown of mouse epidermal skin cancer cell lines resulted in reduced invasion and migration [37]. A heightened level of claudin 1 expression has been found in cervical intraepithelial neoplasia compared to normal squamous cervical epithelial cells [38]. This finding is probably related to different carcinogenesis of these lesions. In cervical cancer HPV infection plays a major role. In this context it is interesting to note that claudin 1 has been found to be a co-receptor for hepatitis virus C and thus promotes viral entry to hepatocytes [39]. Claudin 1 or 7 did not, however, have any association with HPV induced tonsillar squamous cell carcinoma [40].

A peculiar feature of claudin 1 expression was nuclear immunostaining observed in some cases. Claudin 1 nuclear location has previously been reported in melanocytic lesions where its translocation was affected by PKC phosphorylation [41]. Nuclear and cytoplasmic expression of claudin 1 was also detected in nasopharyngeal carcinoma cell lines where it was associated with decreased apoptosis [42].

In contrast to claudin 1, claudin 2 expression was elevated in AK and SCC compared with normal skin. Since claudin 2 has been found to increase paracellular membrane permeability, its overexpression leads to a leakage of barrier. In cervical intraepithelial neoplasia and carcinoma claudin 2 expression was similarly increased [38]. We did not find significant differences in claudin 2 expression between dys-

plasia and invasive carcinoma as was the case in cervical lesions [38]. In glandular epithelial dysplasia such as gastric dysplastic lesions claudin 2 expression is also increased [43]. Both claudin 1 and 2 are elevated in chronic colitis associated dysplasia as well as in related adenomas [44] suggesting that elevated claudin 2 expression with a consequent increase in paracellular leakage may be a feature of dysplasia. It is possibly associated with several molecular pathways which lead to a neoplastic process.

In this study claudin 3 and 5 did not show significant patterns of expression in dysplastic or neoplastic tissues and were mainly expressed in adnexal and vascular structures, respectively. In dysplastic Barrett's esophagus and dysplasia of the esophageal squamous epithelia claudin 3 mRNA was strongly elevated [45] but some authors report increased methylation of the gene leading to claudin 3 silencing [46]. Our results show that claudin 3 is occasionally upregulated in skin SCC but not in AKs or normal skin. This is in line with cervical lesions where no claudin 3 expression was found in normal or dysplastic epithelium [38]. Claudin 5 expression is induced in squamous cell carcinomas of the oesophagus [25] and it may also be expressed in adenocarcinomas such as ovarian carcinomas [18, 47]. Our results show that claudin 5 may occasionally be detected in skin SCC as observed in other epithelial tumors.

For claudin 4, neoplastic skin lesions showed a weaker overall expression compared with normal skin with no significant differences between SCC and AK. Only one case of SCC showed strong positivity indicating that claudin 4 overexpression compared to normal skin is rare in skin neoplasia. This is in contrast with cervical lesions were claudin 4 expression was increased in dysplastic and neoplastic lesions [38]. In gastric dysplasia, claudin 4 is elevated compared to normal mucosa making it a putative marker for a precursor lesion of gastric carcinoma [48]. Similarly, in esophageal dysplastic and neoplastic lesions claudin 4 mRNA and protein expression was elevated compared to non-neoplastic epithelium [45]. Even so, several factors like microRNAs, might influence transcription so that mRNA and protein levels may not correlate with each other. On the other hand, breast in situ carcinomas showed a lower expression of claudin 4 compared to normal tissues [49]. Thus claudin 4 appears to be up- or downregulated in preneoplastic lesions depending on the cell type in question.

Claudin 7 expression was more often present in SCC than in normal skin. Overexpression can not, however, be observed in preneoplastic conditions indicating it to be a late event in the epidermal neoplastic process. In cervix, dysplastic lesions were found to express more claudin 7 [38]. On the other hand, in colorectal columnar mucinous epithelium claudin 7 mRNA and protein expression were decreased in dysplasia and carcinoma compared to normal mucosa [50] while claudin 7 was increased in gastric dysplasia, in oesophageal squamous cell dysplasia and Barrett's oesophagus but weaker expression was detected in adenocarcinoma [45, 51].

In conclusion, our results show notable changes in claudin expression especially with claudin 1, 2 and 7 when comparing normal skin to AK and SCC. These changes may be related to changes in cancer genes during epidermal carcinogenetic process involving for instance p63 expression [35, 52]. On the other hand, claudins may also be affected by immunologic mechanisms. In allergic dermatitis claudin 1 was downregulated which diminished TJ barrier function and induced epidermal cell proliferation [53]. In psoriasis, on the other hand, claudin 1 was elevated leading to increased apoptosis [54]. The expression of other claudins, like claudin 6 or 23 may also play an important role in epidermal disease and neoplasia [53, 55]. Clearly this topic needs further investigation.

Acknowledgements

This study was supported by grants from the Orion-Farmos Foundation, the Thelma Mäkikyrö Foundation, the Finnish Anti-tuberculosis Association, the Finnish Cancer Society and from the Finnish Cultural Foundation. The skillful technical assistance of Erja Tomperi, Mirja Vahera and Aija Parkkinen is appreciated.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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