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Identification of HNP3 as a tumour marker in CD4+ and CD4– lymphocytes of patients with cutaneous T-cell lymphoma

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ABSTRACT

Cutaneous T-cell lymphomas (CTCL) are characterized by malignant proliferation of skin homing T-cells. Although prognosis is generally good, reliable markers are needed to identify patients at risk for a more aggressive course. ProteinChip (SELDI) technology was used as a tool for the discovery of protein patterns in lymphocytes from patients with CTCL ($n = 25$) and unaffected controls ($n = 25$). Lymphocytes were separated in CD4+ and CD4– fractions by magnetic cell sorting (MACS). Each whole protein extract was analysed by ProteinChip technology. The resulting protein profiles were submitted for bioinformatic analysis including a clustering algorithm, a rule extraction, a rating and a rule-base construction step. For the generated combined rule base for the CD4– cell fraction, both the sensitivity and specificity for the prediction of CTCL reached 96%, while for the CD4+ fraction they were 92% and 84%, respectively, for sensitivity and specificity. The most significant peak at 3489 Da could be identified as HNP3, an α-defensin, by immunocapturing. These results open up both the possibility for the use of this protein signature, especially HNP3, to more effectively monitor and screen CTCL, and the avenue to identify the other relevant peaks for a better understanding of the development of this tumour.

1. Introduction

Malignant lymphomas can affect the integument primarily and secondarily. If no extracutaneous manifestations can be detected with routine staging methods, a primary cutaneous lymphoma can be assumed. The special status of cutaneous lymphomas results from the skin associated control circuit of lymphocytes that recycle between skin and lymph node. The majority of cutaneous lymphomas are classified as T-cell lymphomas. More than 90% of the T-cell lymphomas are represented by mycosis fungoides (MF) and the leukaemic variant known as Sézary syndrome (SS). Cutaneous T-cell lymphomas (CTCL) of MF type start mostly in middle adulthood and have an incidence of 0.4/100,000 individuals/year in the US [1] but this is on the increase. CTCL is classified as a peripheral T-cell lymphoma of low malignancy with a

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prolonged indolent course. Prognosis depends mainly on the stage and severity of skin involvement and lymph node status. At the beginning of the disease histological diagnosis can be especially difficult. While life expectancy in patients with skin involvement T1 is not reduced at all, the 10-year survival rate in patients with T3 and T4 is as low as 40%. At this time, clear cut prognosis for an individual case is not possible [2].

Despite enormous efforts only a few tumour disease relevant markers have been established that can be used for early diagnosis or for a better therapy in malignancies [3]. This is despite the fact that new high parallel genomic and proteomic techniques have been established in the last few years. Up to now, there is also no highly specific tumour marker for CTCL although an increase of lactate dehydrogenase (LDH) is associated with a worse prognosis for the patient in tumour stage or erythrodermia [4]. Although numerous potential candidates have been investigated, none are yet used routinely in clinical settings. One candidate marker is neopterin, which are pyrazoino-pyrimidine molecules that are produced after IFN-γ stimulation and indicate activation of the cellular immune response [5]. Neopterins were elevated in patients with higher stages of CTCL's compared to patients with psoriasis and atopic eczema, but not in patients with SS where they were only slightly elevated. Increased neopterin levels are seen in many other malignant diseases and are not specific to CTCL. Another candidate is the α chain of soluble IL-2R (sIL-2R). Its concentration was found to be increased in advanced tumour stages of CTCL. The correlation between sIL-2R and the severity of the skin and lymph node status was better for sIL-2R than for LDH or the β2-microglobulin [6].

For the discovery of new biomarkers at the proteomic level, surface enhanced laser desorption/ionization-mass spectrometry (SELDI-MS)-based ProteinChip technology is one of the most promising techniques [7]. This technology makes use of affinity surfaces to retain proteins based on their physico-chemical characteristics, followed by direct analysis by time of flight mass spectrometry (TOF-MS) [8]. Thus, proteins being retained on chromatographic surfaces can be easily purified from contaminants such as buffer salts or detergents, thus eliminating the need for pre-separation techniques, as required with other MS techniques. Furthermore, the low sample requirements of this technique are ideal for small biopsies, microdissected tissue or cell subfractions of blood [9].

Until now, biomarker discovery with the ProteinChip technology was mostly done by analyzing body fluids like serum or urine, as body fluid analyses are fast and easy to perform by direct application on the arrays. Nevertheless, it is known that intra-individual changes in serum are high. Hence, biomarkers responsible for the genesis and progression of cancer must be present at a high level to be observed above normal changes [10]. CTCL blood samples fractionated by FACS or MACS open up the opportunity to analyze those cells that are most likely involved in tumour genesis and progression.

When specific alterations between the protein profiles are detected by ProteinChip technology, single peaks can be isolated and identified by either 2-DE or ProteinChip technology [11] and by collision-induced dissociation (CID) using a ProteinChip interface coupled to a tandem mass spectrometer [12].

In the study presented here, fractions of CD4+ and CD4− lymphocytes from 25 CTCL patients and 25 normal controls were analysed on ProteinChip Arrays, because it is known that CD4 positive lymphocytes are activated in CTCL. The resulting protein profiles were submitted to a clustering algorithm, a rule extraction and rule base construction step which together excluded the possibility of finding a protein pattern by chance. For the prediction of CTCLs, the generated combined rule base resulted in a specificity of up to 84% and sensitivity of 92% for the CD4+ cell fraction and 96% specificity and sensitivity for the CD4− fraction.

2. Materials and methods

2.1. *Processing of blood samples*

All blood samples of CTCL patients (n = 34) were obtained from the Clinic of Dermatology of the Friedrich-Schiller-University Jena or the Clinic of Dermatology in Dresden, Germany with informed consent. CTCL diagnostics were performed in accordance with the guidelines of the German Cancer Society (AMWF) and were based upon clinical investigations: ultrasound investigations of lymph nodes, spleen and liver; routine laboratory investigations including: a complete differential blood count, Sezary cell count, liver enzymes, lactat dehydrogenase, renal parameters and inflammatory parameters (Westergreen blood sedimentation rate, C-reactive protein); and histology of lesional skin, lymphnodes (when enlarged) and bone marrow (when indicated). In addition to hematoxylin-eosin stains, immunostains and PCR for T-cell receptor were performed. CTCL were classified according to EORTC recommendations. Classification of all evaluable patients, who were all active, are given in Table 1. Blood samples from healthy sex-matched donors were used as a control (n = 30). These normal samples (5 ml) were stimulated with phytohaemagglutinin (PHA) to activate lymphocytes. Blood samples were further processed not longer than 12 h after sampling.

2.2. *Separation of lymphocytes by MACS*

Ten ml of heparinized blood was quartered in aliquots of 2.5 ml, diluted with 2.5 ml phosphate buffered saline (PBS), layered onto 5 ml chilled Biocoll (density 1.077 g/ml, Biochrome AG, Germany) and centrifuged at 800g for 30 min (without brake) at room temperature. After this density gradient separation, the resultant opaque interface containing lymphocytes was carefully transferred with a syringe into a centrifuge tube and washed two times with PBS.

The washed cells were resuspended in 80 μl of PBS and stained with CD4 microbeads (Miltenyi) for 15 at 4 °C. The PBS washed cells were centrifuged at 600g for 10 min, washed and resuspended in 500 μl PBS-FCS. Afterwards the cells were separated on a miniMACS column which was placed in a MACS magnetic field (Miltenyi Biotec GmbH, Germany) according to the manufacturer's instructions. The non-retained cells containing all other lymphocytes except CD4+ is further referred to as the CD4− fraction. The magnetically retained cells were eluted after washing and are designated as the CD4+ cell fraction. Before and after MACS, the cell number was analysed with a Neubauer counting chamber.

Table 1 – Individual staging of evaluable patients and their classification according to EORTC

Patient No.	TNM	EORTC
165	pT2cN0cM0B0	Mycosis fungoides
167	pT4pN3cM0B0	Mycocis fungoides
168	pT3cN0M0B0	Mycosis fungoides
169	pT3cN0cM0B0	Large cell CTCL, CD30-positive
171	pT2cN0cM0B0	Mycosis fungoides
172	pT2cN0cM0B0	Mycosis fungoides
173	pT2N0M0	Mycosis fungoides
174	pT3pN1pM1B0	Mycosis fungoides
175	pT3pN1cM0B0	Mycosis fungoides
176	pT3cN0cM0B0	Mycosis fungoides
177	pT3pN3cM0B0	Mycosis fungoides
178	pT2cN0cM0B0	Mycosis fungoides
181	pT2cN0cM0B0	Mycosis fungoides
182	pT3pN1cM0B0	Mycosis fungoides
184	pT4pN1cM0B0	Mycosis fungoides, erythrodermatic variant
185	pT2cN0cM0B0	Mycosis fungoides
186	pT3cN0cM0B0	Mycosis fungoides
187	pT2cN0cM0B0	Mycosis fungoides
188	pT1cN0cM0B0	Mycosis fungoides
190	pT2cN0cM0B0	Mycosis fungoides
194	pT2cN0cM0B0	Mycosis fungoides
195	pT1cN0cM0B0	Mycosis fungoides
196	pT2cN0cM0B0	Mycosis fungoides
197	pT2cN0cM0B0	Mycosis fungoides
198	pT3N1M0	Mycosis fungoides

2.3. *Profiling of CD4+ and CD4− fractions*

The protein lysates from CD4+ and CD4− lymphocyte fractions were analysed on a hydrophobic reverse phase array (H4; Ciphergen Biosystems Inc., Fremont, CA) as described elsewhere [13]. In brief, array spots were preincubated with 5 μl acetonitrile and rinsed with deionised water. Then 2 μl of sample extract were spotted on ProteinChip Arrays and allowed to dry. After washing three times with the binding buffer, two times 0.5 μl sinapinic acid (saturated solution in 0.5% TFA/50% acetonitrile) was added and mass analysis was performed in a ProteinChip Reader (PBS-II, Ciphergen Biosystems Inc., Fremont, CA) according to an automated data collection protocol.

2.4. *Bioinformatic analysis of ProteinChip Array data*

The resulting protein profiles between 2 and 20 kDa were subjected to CiphergenExpress (CE) 3.0 software and a cluster and rule-based data mining algorithm (XL-Miner 3.0, BioControl). The CE software was used for the processing of raw spectra and the calculation of *P*-value and cluster plots according to the manufacture's instruction. The data analysis process with XL-Miner consists of a clustering step, a rule extraction and rating step, and a rule-base construction step as described elsewhere [14]. All these steps are supervised with respect to the given sample classification (CTCL vs. unaffected). Log 2-transformed and normalized data were clustered in a supervised mode into two clusters – "low expressed" and "high expressed" – for each peak using a modified fuzzy c-means algorithm [15]. In rule-extraction rules are generated and rated by a statistically based rule rating measure introduced by Kiendl and Krabs [16]. Finally, a small subset of rules from the rule list can forms a rule base that can be used for the automatic classification of new patient samples. To classify a new patient sample, the cluster memberships (condition part of the rules) of all rules from the rule base that point to the same classification outcome (conclusion part of the rules) are added and the sample is assigned to the class with the highest sum.

2.5. *Identification of differentially expressed protein peaks*

For immunocapturing, 3 μl (60 ng) of anti-human monoclonal antibody for α-defensin 1–3 (HNP1–3; T-1034; BMA Biomedicals; Augst, Switzerland) were incubated with 10 μl protein A-agarose (Sigma) for 15 min on ice. The sample was centrifuged and the supernatant was discarded. Thereafter, the resulting sediment was incubated with blocking solution containing 2% milk powder for 30 min on ice. The supernatant was discarded and the pellet was washed three times with a buffer (COIPB) containing 20 mM Hepes (pH 7.8), 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS. Afterwards, 5 μl of a lysate from CTCL CD4− cells were incubated with this pellet for 1 h on ice. As a negative control an unspecific antibody (IgG rabbit) was coupled to the protein A-agarose and treated in the same way. After incubation, samples were washed three times with COIPB. The proteins were eluated in 12 μl elution buffer and 3 μl of supernatant were analysed on SAX2 ProteinChip Array.

3. Results

3.1. *Separation of lymphocytes by MACS*

The density centrifugation resulted in 10^5–10^7 lymphocytes. The resulting cell numbers were strongly dependent on the time between blood collection and the further processing.

After MACS, the cell numbers for CD4+ and CD4− cells was between 10^4 and 10^5.

3.2. Profiling of CD4+ and CD4− fractions

For this study, sample of both CD4+ and CD4− fraction from CTCL and normal controls were applied to H4 arrays and analysed on a PBS-II system. In the low range (2–20 kDa) up to 155 peaks were detected with normalized intensities with a S/N of 3. Spectra with corresponding to low peaks were excluded, so that 25 patients samples (Table 1) and 25 control samples were further analysed. After evaluation, CiphergenExpress software data were exported in a csv-file format for bioinformatic processing.

3.3. Bioinformatic analysis

The *P*-values of all detected peaks were calculated by the CiphergenExpress software. For the CD4− fraction the best value (3.05×10^{-7}) was found for the peak at 3490 Da and for the CD4+ lymphocytes at 8565 Da (1.7×10^{-6}). Distribution of intensities for these peaks for controls and patients with CTCL are shown in Figs. 1 and 2.

The cluster and rule based data mining method (XL-miner) described above revealed six peaks for the CD4− fraction (Fig. 3, Table 2) whose combination is relevant to distinguish between unaffected or patients with CTCL and five such peaks for the CD4+ fraction (Fig. 4, Table 3). After combining the generated rules, the calculated sensitivity and specificity was 96% for the CD4− fraction. For the CD4+ lymphocytes a sensitivity of 91.7% and a specificity of 84% was revealed.

3.4. Identification of HNP3

To confirm that HNP3 are matching to the differentially expressed peak at 3489 Da identified by ProteinChip analysis an immunoassay was performed using monoclonal antibodies against HNP1–3. In this procedure, a specific anti-HNP1–3 antibody bound on protein A-agarose captured α-defensin 1–3 from the CTCL patients' CD4− cell fractions. The captured proteins were eluted from the beads and applied to an SAX2 ProteinChip for analysis by SELDI-MS. The spectra of the analysis showed peaks corresponding to HNP1–3. In a control assay using protein A-agarose beads without a specific antibody no proteins specific for HNP1–3 was captured (Fig. 5).

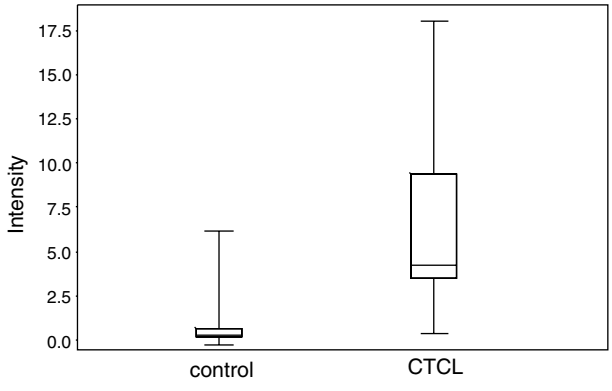

Fig. 1 – Distribution of intensity values of the protein peak at 3489 Da (later identified as HNP3) for the CD4− fraction from patients and normal controls ($P = 3.05 \times 10^{-7}$).

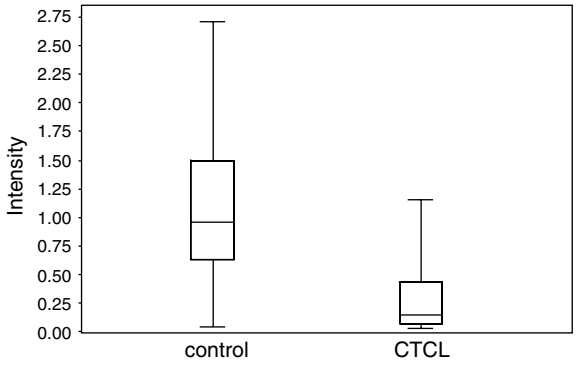

Fig. 2 – Distribution of intensity values of the protein peak at 8565 Da for the CD4+ fraction from patients and normal controls ($P = 1.7 \times 10^{-6}$).

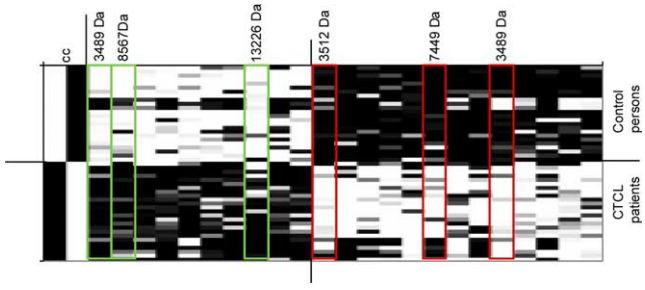

Fig. 3 – Rule list (α = 95) with relevant rules (see Table 2) for the prediction of CTCL (red frame) and normal controls (green frame) of the CD4− cell fraction. Samples are clustered horizontally, peaks vertically. The specificity of the combined rule base is 95.8%, the sensitivity 96%. The peak with 3489 Da was identified as HNP3. cc: clinical classification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

New biomarkers or biomarker patterns found by genomic or proteomic high-through put techniques should enable scientists and medical staff to make more reliable early diagnoses of certain human diseases, especially malignant tumours, and to facilitate the prediction of their progression. In this way, biomarkers could contribute to a more differentiated, individually orientated tumour therapy. Despite enormous efforts only a few relevant markers have been presently established for tumour diseases [3]. For CTCL it would be desirable to have a serum parameter that could be used for diagnosis and, more importantly, enable clinicians to monitor the course of the disease during therapy.

One of the most promising proteomic tools for the detection of new proteomic cancer biomarkers is Ciphergen's ProteinChip technology [10]. This technique has been

Table 2 – Rule base for the CD4− cell fraction to distinguish between CTCL patients and normal controls

Condition (IF)	Conclusion (THEN)
Expression at peak 3489 Da high (>2.07)	CTCL
Expression at peak 3512 Da high (>0.52)	CTCL
Expression at peak 7449 Da high (>1.76)	CTCL
Expression at peak 3489 Da low (<1.39)	Normal
Expression at peak 8567 Da high (>0.09)	Normal
Expression at peak 113,226 Da low (<3.96)	Normal

All expression values are log-2 transformed. The specificity of the combined rule base is 95.8%, the sensitivity 96% (3489 Da was identified as HNP3).

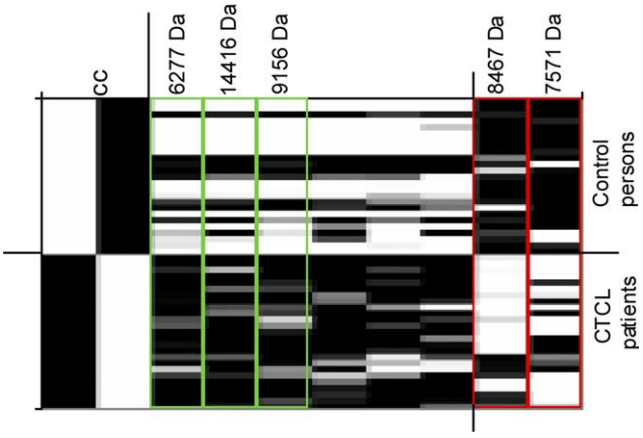

Fig. 4 – Rule list (α = 95) with relevant rules (see Table 3) for the prediction of CTCL (red frame) and normal controls (green frame) of the CD+ cell fraction. Samples are clustered horizontally, peaks vertically. The specificity of the combined rule base is 84%, the sensitivity 91.7%. cc: clinical classification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predominantly used for body fluids, as they are fast and easy to analyse by direct application on the ProteinChip Arrays. Despite the relatively high inter- and intra-individual changes in serum, a large number of studies using body fluids as starting material have been published on serum or other body fluids [17]. This pursuit for markers is motivated by the realization that if they can be found through bioinformatics processing they would be ideal for screening high-risk individuals or even individuals without elevated risk, as discussed by Kozak and co-workers in a study of ovarian cancer [18] and others as well.

Table 3 – Rule base for the CD4+/− cell fraction to distinguish between CTCL patients and normal controls

Condition (IF)	Conclusion (THEN)
Expression at peak 7571 Da high (>−2.52)	CTCL
Expression at peak 8565 Da low (<−2.72)	CTCL
Expression at peak 6277 Da high (>0.51)	Normal
Expression at peak 9156 Da high (>−1.95)	Normal
Expression at peak 14,416 Da high (>−3.17)	Normal

All expression values are log-2 transformed. The specificity of the combined rule base is 84% and sensitivity 91.7%.

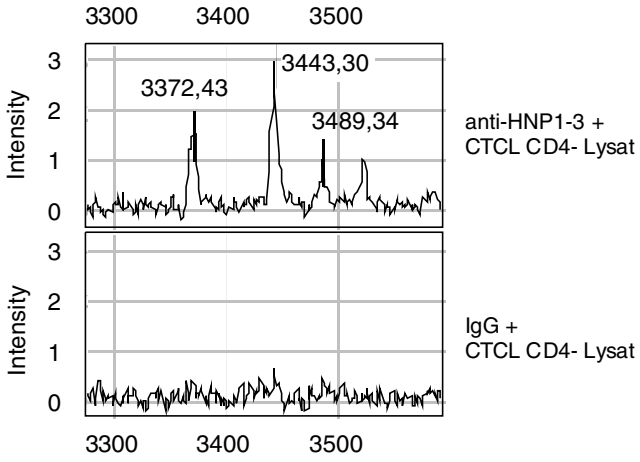

Fig. 5 – Normalized ProteinChip Arrays profiles of the immunocaptured assays of CTCL CD4− cell lysate. For HNP1–3 identification, CTCL a CD4− cell lysate was used as the starting material for immunocapturing assays using the corresponding monoclonal antibody bound on protein A-agarose. The peaks at 3.37, 3.44, and 3.48 representing HNP1–3 were clearly detectable in samples eluted from the protein A-agarose. In control assays with an unspecific antibody no HNP1–3 were captured.

In contrast to serum, the analysis of tissues or fractionated cells is more time consuming as microdissection or cell sorting is necessary. However, the chance to find a reliable tumour marker might be higher than in serum. At the very least, there is higher probability to obtain more information about the biological mechanisms leading to the genesis and progression of cancer [19,20].

Diseases like Mycosis fungoides and Sezary syndrome have already been investigated with the technique of cDNA-Array [21]. Herein, we have demonstrated that the method of MS can be applied to distinguish at the protein level between CTCL patients and control persons.

Intriguingly, no studies analyzing blood fractions have been reported in the analysis of these tumours, although this seems a more promising way using either tissue or even total serum. Especially in CTCL this seems the best choice to gain T-lymphocytes that could not easily be isolated from skin.

In our study, we therefore did not analyse serum, but nucleated cells from blood samples. As a separating method we used magnetic bead-coupled antibody against CD4, because it is known that CD4 positive lymphocytes are activated in CTCL. As control, we have therefore used PHA-activation (to stimulate lymphocytes) of the control samples to compare CTCL patients and control persons. In this way, we achieved isolation of a CD4 positive fraction containing T-lymphocytes and a CD4 negative fraction containing all other nucleated cells (e.g., granulocytes). Data from the ProteinChip analysis were submitted to bioinformatic tools to obtain a classifier or a signature to differentiate between patients and normal controls. The results gave rise to a signature of five to six proteins, which could classify the two groups (CTCL CD4− vs.

PHA activated CD4− controls) up to 96%. This is, to the best of our knowledge, the most suitable marker for CTCL so far. Interestingly, the CD4− fraction revealed the most significant results. This might be due to the extraordinary good predictor at 3489 Da.

In a former study, we had shown that colorectal cancer include HNP1–3 [22]. The protein pattern also generated using ProteinChip Technology SELDI TOF MS was identical in this mass range to the protein pattern derived from CTCL. According to this information, we investigated the patients' material for HNP3 expression and precipitated a protein with a mass of 3489 Da with an monoclonal antibody against HNP1–3.

This peak, identified as HNP3 belongs to defensins, which are small antimicrobial peptides that contain six cystein residues which form three disulfide bonds. On the basis of the position of these residues α- and β-defensins have been classified. So far six α-defensins have been identified in humans (human neutrophil proteins 1–3 [HNP1–3] and human defensins 5 and 6 [HD-5 and -6]), but many more probably exist [23]. HNP1–3 may also be expressed in intestinal epithelial cells under certain conditions. In a recent study, expression of HNP1–3 was observed in epithelial cells of the ileum and colon in cases of inflammatory diseases but not in normal intestinal tissue [24]. Whether this reflects the induction of gene expression or the uptake by epithelial cells of peptides released by neutrophils in the vicinity remains to be determined [25]. In different cancer entities like renal cell carcinoma (RCC) [26] or oral squamous cell carcinoma (OSCC) [27], HNP1–3 were also found to be up-regulated. Defensins may play a further role in adaptive immunity given that HNP1 and -2 are chemotactic for human T cells both in vitro and in vivo [28]. Both α- and β-defensins have the capacity to chemo-attract immature dendritic cells [29]. Overall, defensins seem to play, besides their antimicrobial function, an important and up to now unclear role in immunity and in progression of tumours [30]. The behaviour of α-defensins might be also crucial in CTLC, but further investigations have to be performed to obtain deeper insights into their particular role in lymphoma. So far, this is the first study to demonstrate the presence of human α-defensins 1–3 in CTCL patients. The identification of the other relevant proteins found in the signature for CD4+ and CD4− fraction is in progress and might contribute to our further knowledge on the biology of CTCL.

It is possible that the signature detected was generated by chance considering the ratio of features to patients. However, this is unlikely given that the two bioinformatic tools we employed used conservative algorithms designed to avoid finding significance in random associations. Still, further analysis must be performed and it should also be investigated whether or not the protein signature we found, especially HNP3, is present in patient serum, as this would greatly facilitate adoption of this marker in clinical use. In conclusion, we have established a promising procedure combining ProteinChip technology and bioinformatic tools that allows a screening for CTCL with high sensitivity and specificity. The specific proteomic signatures described here, even without the knowledge of all respective proteins, can serve as an additional diagnostic parameter for a better insight into the genesis and progression of CTCL.

Conflict of interest statement

None declared.

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