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ORIGINAL INVESTIGATION

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Evidence for differential *S100* gene over-expression in psoriatic patients from genetically heterogeneous pedigrees

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Abstract Psoriasis is an inflammatory skin disorder characterised by keratinocyte hyper-proliferation and altered differentiation. To date, linkage analyses have identified at least seven distinct disease susceptibility regions (PSORS1–7). The PSORS4 locus was mapped by our group to chromosome 1q21, within the Epidermal Differentiation Complex. This cluster contains 13 genes encoding S100 calcium-binding proteins, some of which (*S100A7*, *S100A8* and *S100A9*) are known to be up-regulated in individual patient keratinocytes. In this study, we analysed *S100* gene expression in psoriatic individuals from families characterised by linkage studies. We first selected individuals from two large pedigrees, one of which was linked to the 1q21 locus, whereas the other was unlinked to that region. We studied the expression of 12 *S100* genes, by semi-quantitative RT-PCR and Northern blot. These analyses demonstrated up-regulation of *S100A8*, *S100A9* and, to a lesser extent, *S100A7* and *S100A12*, only in the 1q21 linked family. We subsequently analysed *S100A7*, *S100*A8, *S100A9* and *S100A12* in three additional samples and were able to confirm *S100A8*/*S100A9*-specific over-expression in 1q-linked pedigrees. Thus, our data provide preliminary evidence for a locus-specific molecular mechanism underlying psoriasis susceptibility.

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Introduction

Psoriasis (OMIM#: 177900) is a chronic inflammatory skin disorder affecting approximately 2% of the Caucasian population (Nevitt and Hutchinson 1996). The disease is characterised by the occurrence of skin lesions showing epidermal hyper-proliferation, abnormal keratinocyte differentiation and infiltration of inflammatory elements (Barker 1991). Familial clustering of psoriasis is well established (Hellgren 1967), and epidemiological data are consistent with the model of a polygenic disorder caused by high-frequency disease alleles (Elder et al. 1994). Psoriasis susceptibility loci have been assigned to several chromosome regions (PSORS1–7), although independent replication of linkage is so far available only for the PSORS1 (6p21), PSORS2 (17q) and PSORS4 (1q21) regions (Elder et al. 2001). The PSORS4 susceptibility locus was assigned by our group to a region containing the Epidermal Differentiation Complex (EDC, OMIM#: 601588) (Capon et al. 1999), a cluster of evolutionarily related genes affecting terminal differentiation of human epidermis (Mischke et al. 1996). Several EDC genes, including involucrin, loricrin, and the three classes of small proline-rich proteins (SPRR), encode structural proteins of the human epidermis (Marenholz et al. 1996). In addition, 13 genes of the S100 family of EF-hand calcium binding proteins also reside within the EDC (Mischke et al. 1996). S100 proteins have been implicated in the calcium-dependent regulation of a variety of intracellular activities such as cell cycle progression, differentiation and inflammation (Donato 2001). Several disorders have been associated with an altered expression of *S100* genes (Marenholz 1996), and studies of isolated patients showed that *S100A7*, *S100A8* and *S100A9* are markedly up-regulated in psoriatic skin lesions (Madsen et al. 1991; Hardas et al. 1996; Bowcock et al. 2001). On the other hand, the relationships between psoriasis susceptibility loci and

EDC gene over-expression have never been investigated. In this study, we have analysed the expression pattern of *S100* genes in a sample of psoriatic patients whose families had been characterised by linkage analysis. We observed an up-regulation of *S100A8* and *S100A9* genes only in the 1q21 linked families, suggesting that the PSORS4 susceptibility gene might cause the disruption of a specific cellular pathway.

Materials and methods

Patients and tissue specimens

Biopsies were taken from psoriatic plaques of patients 443, 452, 769, 774 and 778, all belonging to large Italian pedigrees, ascertained from the medical records of the IDI institute. Consensus diagnosis of the disease was carried out by two expert dermatologists, who obtained patients' informed consent for their involvement in this study. Linkage to chromosome 1q had been demonstrated for families of patients 443, 769 and 774, by typing D1S305, D1S2346, 140J1D, D1S498 and D1S1664 markers. Conversely, the analysis of D6S258, D6S265, and D6S306 microsatellites had demonstrated that the family of patients 452 and 778 was in linkage with chromosome 6p21. In all pedigrees posterior probabilities of linkage >0.8 were observed. Ten further skin biopsies, sampled during routine plastic surgery of unaffected individuals, were used as controls. Biopsies were processed for primary keratinocyte culture and cells were grown on a feeder-layer of lethally irradiated 3T3-J2 murine fibroblast (gift from H. Green, Harvard Medical School, Boston, Mass.) as described elsewhere (Zambruno et al. 1995). All cultures were subjected to three passages and harvested when they reached 90% confluence.

Semi-quantitative RT-PCR

Total RNA was prepared by a standard acid guanidium/thiocyanate/phenol-chloroform protocol (Chomczynski and Sacchi 1987). RNA integrity and concentration were verified by agarose-gel electrophoresis, and aliquots of 0.5 µg were sampled to carry out oligo(dT)-primed reverse transcription, using Gibco BRL (Gaithersburg, Md.) reagents and recommendations. *S100* transcripts were amplified using primer pairs derived from the corresponding GenBank sequences (see Table 1). As *S100A5* is expressed only in restricted areas of the brain (Schafer et al. 2000), this gene was not included in our study. In order to maximise the specificity of PCR reactions, all primers were designed outside the regions of *S100* gene homology. Primers amplifying a control fragment from the glucose phosphate isomerase (*GPI*) house-keeping gene were included in each reaction (Table 1). PCR conditions were as follows: three serial dilutions of each cDNA were amplified in a 25-µl mix containing 1×reaction buffer (Promega, Madison, Wis.), 175 µM dNTPs, 15 pmol of each primer, 1 U Taq DNA polymerase (Promega, Madison, Wis.). Reactions were cycled for 25 times at 94 °C for1 min, 55 °C for 1 min, 72 °C for 1 min. When the reaction was complete, 15 µl of each amplified product were loaded on a 2% agarose gel containing 1 µg/ml ethidium bromide. Gel images were saved with a gel doc 1000 UV camera (Biorad, Hercules, Calif.) and the *S100/GPI* ratio was quantified using the Imagequant 1.1 software (Molecular Dynamics, Little Chalfont, UK). S100/GPI mean ratios from different samples were compared using SigmaStat software 1.0 (Jandel Scientific, Erckrath, Germany) to run the *t*-test and ANOVA.

Northern blot analysis

Samples of total RNA (20 µg) were size-fractioned by electrophoresis on 1% formaldehyde-agarose gels and blotted to nylon membranes, by standard methods. Gene specific probes were obtained by RT-PCR, using the *S100* and *GPI* primers listed in Table 1. Amplified products were gel-purified using the Ultrafree-DA kit (Millipore, Bedford, Mass.) and labelled with ^{32}P-dCTP, using the Rediprime kit (Amersham, Little Chalfont, UK). Each *S100* probe was sequentially hybridised, with *GPI*, on a different blot, under stringent conditions. Quantitative analysis of autoradiographs was carried out as described above.

Nomenclature

Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001)

Results and discussion

In a first phase of this study, we analysed the expression of 12 *S100* genes in samples 443 (originating from a 1q-linked pedigree), 452 (originating from a 1q-unlinked pedigree) and CTR1–10 (taken from unrelated control individuals). Sample 443 demonstrated a marked up-regulation of *S100A8* (P=0.009) and *S100A9* (P=0.001) genes,

Table 1 RT-PCR primer sequences

Target gene *(Accession number)*	Forward primer (5′→3′)	Reverse primer (5′→3′)
S100A1 *(X58079)*	CCACACACAGCTCCAGCAGCC	GCTTGGACCGCTAGTCTTGCGCC
S100A2 *(M87068)*	GGAGCAGGCGCTGGCTGTGC	CCTGGGCCCAAGAGATCCATGG
S100A3 *(Z18948)*	CCGAACTGGTCAACTCTCAAGAGACC	CGCTCTGCTGAGCCTCGAGGGC
S100A4 *(M80563)*	CCCCTCTCTACAACCCTCTCTCC	GCACGTGTCTGAAGGAGCCAGG
S100A6 *(J02763)*	CCTCGACCGCTCGCGTCG	CCAGAGGGTGTCTCCATCTTCC
S100A7 *(M86757)*	TTCTACTCGTGACGCTTCC	GACATTTTATTGTTCCTGGGTCTC
S100A8 *(M21005)*	GCTGTCTTTCAGAAGACCTGG	CTCTGGGCCCAGTAACTCAG
S100A9 *(M21064)*	CTCTGTGTGGCTCCTCGGCTTTGG	CCAGCCCCTAGCCCCACAGCC
S100A10 *(M38591)*	CCACTCCGCTGCTCGCC	CCTGATCTGCTCATGAAATCC
S100A11 *(D38583)*	GGGCAAGGCTGGGCCGGG	TATTGGCAGGTGGGGCCTGC
S100A12 *(X97859)*	GCTCCACATTCCTGTGCATTGAGG	CCCTCATTGAGGACATTGCTGGG
S100A13 *(X99920)*	GGTCAGCTAGCCCCTTGAGG	GGGAAGAGTGCGGTTCTGC
GPI *(AH002710)*	GCAGTGGCGAAGCACTTT	ACAATAGAGTTGGTTGGGCG

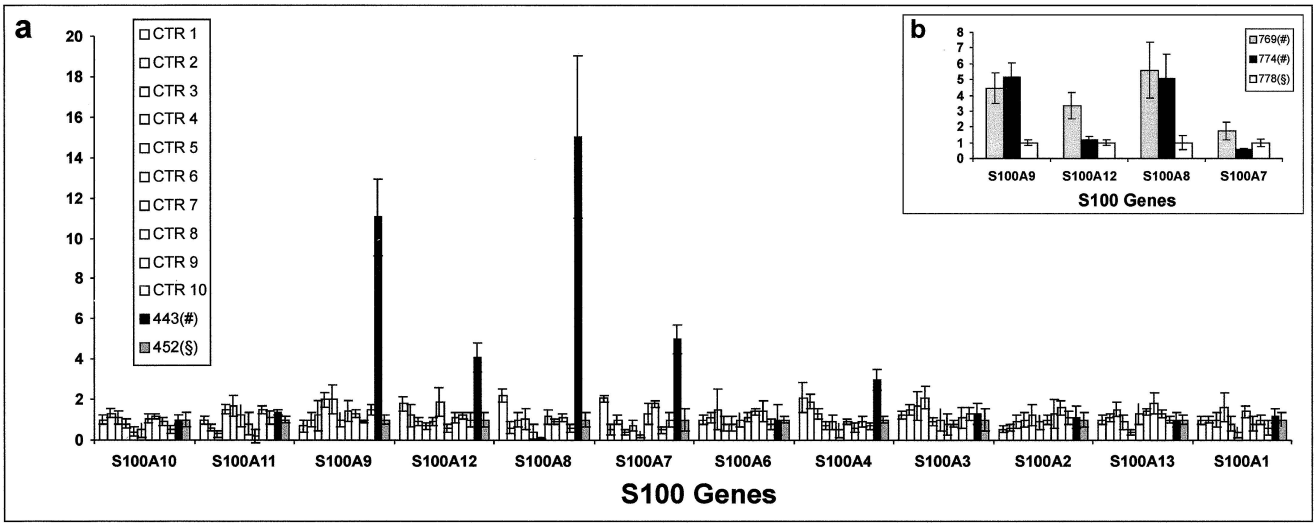

Fig. 1a, b Histograms of mean *S100* gene levels relative to glucose phosphate isomerase (*GPI*), determined by densitometric analysis of RT-PCR products. The x-axes reproduce the organisation of the EDC, as expression levels of clustered genes are plotted sequentially. **a** First set of samples; **b** second set of samples (# samples originating from 1q-linked pedigrees; § samples originating from 1q-unlinked pedigree)

as well as a marginal increase of *S100A7* (P=0.03) and *S100A12* (P=0.04) expression (Fig. 1a). At the same time, samples CTR1–10 demonstrated no significant variation in *S100* levels, as shown by ANOVA analysis (P>0.05).

In order to confirm these data, 12 Northern blots were prepared using RNAs from 443, 452 and CTR1–10 samples, and each filter was sequentially hybridised with *GPI* and an *S100* specific probe. In all cases, the observed *S100/GPI* ratios were consistent with those determined by semi-quantitative RT-PCR (Fig. 2a).

We sought a further confirmation of our results by analysing *S100A7*, *S100A8*, *S100A9* and *S100A12* expression, in three additional samples originating from 1q-linked (769, 774) and 1q-unlinked (778) pedigrees. Both RT-PCR and Northern blot analysis confirmed the up-regulation of *S100A8* (P=0.008) and *S100A9* (P=0.007), but failed to validate *S100A7* and *S100A12* putative over-expression (P>0.05) (Figs. 1b and 2b). Thus our survey failed to detect any convincing evidence for the *S100A7* up-regulation reported by other authors (Hardas et al. 1996; Bowcock et al. 2001). The putative over-expression observed in sample 443 was supported by a P value of borderline significance, so that the failure to validate this observation in the second set of samples was not unexpected. The discrepancy with published data on *S100A7* expression may be due to differences in the pathogenesis of familial and isolated forms of psoriasis. Besides, some degree of variability in *S100* gene up-regulation would not be unexpected in a disease, which is characterised by a wide spectrum of clinical severity.

In contrast, our analysis clearly confirmed the significant over-expression of the *S100A8* and *S100A9* genes, but only in the biopsies originating from 1q21 linked families. We are aware that these data were obtained in a small sample and the analysis of additional individuals from 1q-linked and unlinked families would be necessary to generalise our results. However, we included in this survey all the extended pedigrees from our patient cohort, deeming as problematical the recruitment of additional multiplex families: psoriasis being a multifactorial disorder, pedigrees that are large enough to allow unambiguous assignment of susceptibility loci are exceedingly rare. We also acknowledge that the modest size of our sample cannot be fully compensated by the many replicates of our experiments. However we believe that the validation obtained on the second data set demonstrates the reliability of our experimental approach and provides preliminary evidence for a locus-specific over-expression of *S100A8* and *S100A9*. Remarkably, these two genes map within a

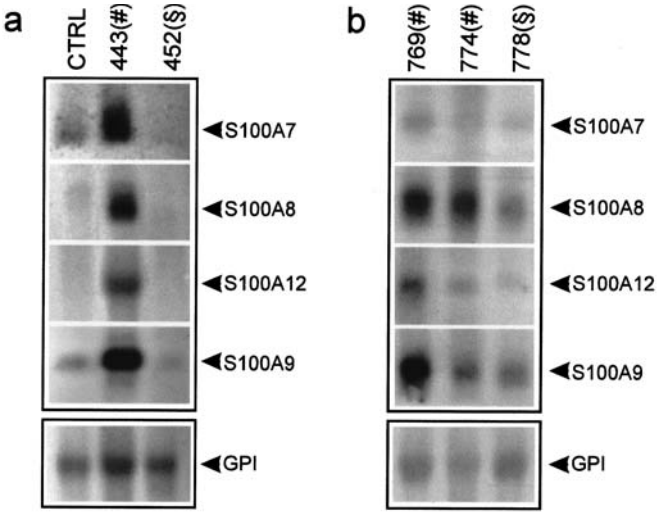

Fig. 2a, b Sequential hybridisation of distinct Northern blots, showing *S100A7*, *S100A8*, *S100A12* and *S100A9* transcript levels relative to *GPI*. **a** First set of samples; **b** second set of samples (# samples originating from 1q-linked pedigrees; § samples originating from 1q-unlinked pedigree)

50-kb genomic segment (South et al. 1999), so that their co-ordinate up-regulation strongly suggests the existence of a locus control region (LCR) whose alteration might contribute to psoriasis onset in 1q-linked pedigrees. This hypothesis would be consistent with the biological role of S100A8 and S100A9. These two proteins can form heterodimers that interact with the cellular receptor for advanced glycation end products (RAGE), triggering cellular activation and generation of key pro-inflammatory elements (Hofmann et al. 1999). Interestingly, up-regulation of *S100A8* and *S100A9* was also observed in the hyperthickened epidermis of human wounds, suggesting that the expression levels of these two genes may affect the balance between keratinocyte proliferation and differentiation, which is typically altered in wound healing and psoriasis (Thorey et al. 2001).

Thus, further investigations of *S100A8/S100A9* function and expression may contribute to define part of the cellular pathways leading to psoriasis onset.

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Electronic database information

URLs for the data in this article are as follows:

- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim
- GenBank, http://www.ncbi.nlm.nih.gov/Genbank

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