SCCA2-like Serpins Mediate Genetic Predisposition to Skin Tumors

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ABSTRACT

Reasons for early onset skin cancer are poorly understood. Microarray analysis revealed overexpression of the Scca2 gene in the 12-O-tetradecanoylphorbol-13-acetate-treated skin of Car-S mice, or line phenotypically selected for high susceptibility to two-stage skin carcinogenesis, as compared with 12-O-tetradecanoylphorbol-13-acetate-treated skin of Car-R mice, which is resistant. A human squamous cell carcinoma cell line (NCI-H520) transfected with mouse Scca2 or a related gene, Scca2-rs1, both expressed in the skin, showed significantly increased tumor growth as compared with controls when injected in nude mice. Immunohistochemical analysis of samples from two independent series of Italian and Korean patients with squamous cell carcinoma of the skin indicated a significant association between SCCA2 protein expression and younger age at tumor onset. These findings provide evidence that SCCA2-like serpins mediate genetic predisposition to skin cancer in a mouse model and in humans.

INTRODUCTION

SCC is a tumor marker, as well as the effects of the serpins in cancer-related development. In the present study, cDNA microarray analysis of TPA-treated skin of Car-S and Car-R mice indicated overexpression of the Scca2 gene in Car-S mice. Scca2 belongs to the serine protease inhibitor (serpin) superfamily, which includes >500 members (reviewed in Ref. 11). Members of this superfamily regulate protease activities associated with inflammation (12), cell migration (13), differentiation (12), and apoptosis (11–14). The SCC antigen is a serological marker of SCC derived from various organs (15–17). The role of the SCC antigen, as a tumor marker, as well as the effects of the serpins in cancer-related phenotypes, prompted us to perform functional studies on the mouse Scca2 gene and to analyze the role of Scca2 protein expression in human skin cancer.

MATERIALS AND METHODS

Mice and RNA Extraction. Adult Car-R and Car-S mice (generation N13) were treated with 1 μg of TPA twice a week for 4 weeks; 2 days after the last treatment, mice were sacrificed, and the skin was excised and frozen. Skin tissue from 3 mice of each line were collected and pooled for microarray analysis. Additional mice, treated in the same way, were used for Northern blot and RT-PCR analyses. Adult male CD-1 nude mice were purchased from Charles River (Calco, Italy).

Total RNA was prepared from mouse skin according to the guanidine thiocyanate protocol (18). Polyadenylated RNA was obtained using the Micromax mRNA isolation kit (Miltenyi Biotec, Auburn, CA).

Array Hybridization and Northern Blot Analysis. Polyadenylated RNAs (1 μg) from TPA-treated skin of Car-R and Car-S mice were reverse-transcribed using random nonamers and Superscript reverse transcriptase (Life Technologies, Inc.). Samples were labeled with the fluorescent dyes Cy-3 and Cy-5 (Amersham Pharmacia) and used as probes on a mouse full-length cDNA 20K microarray set (19). Arrays were laser-scanned using ScanArray 5000 (GSI Lumronics), and data were analyzed using the program ScanAlyze, followed by a filtering procedure (20).

Northern blots were prepared using 20 μg of total RNA from each sample of TPA-treated skin of Car-R and Car-S mice. RNA was electrophoresed on a 1% agarose denaturing gel, transferred to nylon membranes, and hybridized with RT-PCR-prepared DNA fragments of Scca2 (nt 469–963 of GenBank clone NM_009126) or the 18S RNA housekeeping control (nt 540–1740 of GenBank clone H9262). The 18S RNA probe was labeled with [α-32P]dCTP by random

Received 10/10/02; accepted 2/19/03.

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1 Supported in part by grants from Associazione e Fondazione Italiana Ricerca Cancro (AIRC and FIRC) of Italy, and by grants from the European Commission (Association Contract No. F14PCT950008a).

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5 The abbreviations used are: NSCC, squamous cell carcinoma; TPA, 12-O-tetradecanoylphorbol-13-acetate; Scca2, squamous cell carcinoma antigen 2; RT-PCR, reverse transcription-PCR; nt, nucleotide; LD, linkage disequilibrium.

Korean patients with squamous cell carcinoma of the skin indicated a significant association between SCCA2 protein expression and younger age at tumor onset. These findings provide evidence that SCCA2-like serpins mediate genetic predisposition to skin cancer in a mouse model and in humans.

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primer synthesis using the Decaprime DNA labeling kit (Ambion, Austin, TX), and hybridizations were carried out at 60°C in 5× saline-sodium phosphate-EDTA, followed by washing with 1 × SSC and 0.1% SDS at 60°C.

LD. Car-R and Car-S mice (generation N16) were genotyped by PCR analysis of microsatellite markers. Spleen DNA was used as template, whereas primers were purchased from Research Genetics (Huntsville, AL). Radioactive [α-32P] (D1Mit137, D1Mit187, D1Mit441, D1Mit338, D1Mit387, D1Mit309, D1Mit87, D1Mit258, D1Mit141, and D1Mit515) and silver-stained (D1Mit10, D1Mit45, D1Mit136, D1Mit135, D1Nds7, and D1Mit163) PCR reactions were carried out in a Perkin-Elmer 9600 or 9700 Thermocycler. PCR products were analyzed by electrophoresis on 6–10% nonreducing polyacrylamide gels. Silver staining was carried out by placing the gel in a 10% ethanol and 0.5% acetic acid solution for 6 min followed by 10 min in 0.1% silver nitrate. After an immediate brief wash in distilled water, the gel was placed in a solution of 1.5% NaOH and 0.15% formaldehyde for at least 10 min.

Cloning and Transformation. cDNA was synthesized from pooled RNA of the TPA-treated skin of 3 Car-R and 3 Car-S mice, using Thermostart reverse transcriptase (Life Technologies, Inc.). The full-length coding region of Scca2 mRNA (GenBank accession no. NM_009126) was PCR-amplified from Car-R and Car-S mice, using a forward primer including 20 nt upstream of the ATG codon and a KpnI restriction site (5'-CACAGGTACCGGCCCCAGG-GAGGAGATCTCGCAAGA-3'), and a reverse primer containing an Apol restriction site and ending just ahead of the TGA stop codon (5'-CACACGGTCGAGGCACCTAGGAGCCACA'3'), and a reverse primer containing an Apol restriction site and ending just ahead of the TGA stop codon (5'-CACACGGTCGAGGCACCTAGGAGCCACA'3'). AGCCACCA, and a reverse primer containing an

Rps18

was produced using the GCG software package (Wisconsin Package Version 10.2, Genetics Computer Group, Madison, WI).

NCI-H520 (American Type Culture Collection, Rockville, MD) cells, derived from a human SCC, were transfected using Superfect reagent (Qiagen) and aligned using the GCG software package (Wisconsin Package Version 10.2, Genetics Computer Group, Madison, WI).

Identification of Scca2 and Scca2-rs1 Sequences in Car Mice. RIKEN cDNA microarrays (20K; Ref. 24) were examined for gene expression differences between Car-R and Car-S mice TPA-treated skin. Several genes (to be reported elsewhere) showed higher expression levels in Car-S as compared with Car-R mice. On the basis of differences in expression levels (~5-folds), involvement in tumorigenesis as a tumor marker, and the availability of antibodies against the human protein, we have selected the Scca2 gene for additional studies. Microarray results were confirmed by Northern blot analysis, which indicated Scca2 transcript levels to be ~10-fold higher in Car-S than Car-R TPA-treated skin (Fig. 1).

In Vivo Tumor Growth Assay. Groups of 10–20 nude mice were injected s.c. into the right dorsal region with 4 × 106 NCI-H520 cells transfected with Scca2, Scca2-rs1, or vector. Mice were examined weekly, and tumor size was measured by a Vernier caliper. The experiment was ended at 10 weeks after injection.

Patients and Immunohistochemistry. Paraffin-embedded samples from 88 patients with skin SCC available at the Istituto Nazionale Tumori (Milan, Italy) were analyzed. A tissue array consisting of 59 specimens of resected SCCs of skin and other tissues from Korean patients was purchased from SuperBioChips Lab. (Seoul, Korea).

Tissue slides in 5 mm citrate buffer (pH 6.0) were preincubated at 95°C in an autoclave for 15 min, and mixed with antibody (101C12 purified mouse anti-Scca2 monoclonal antibody; Ref. 21), used at a concentration of 1:50, and standard avidin-biotin complex. Immunostaining was scored as present or absent. Two independent pathologists scored the results without knowledge of any clinical data.

Statistical Analysis. Fisher’s exact test was used to evaluate LD for segregation of marker alleles in Car-R and Car-S lines, and tumor incidence in nude mice. The univariate analysis of variance procedure was used for analysis of tumor growth curves in nude mice, followed by pairwise multiple comparisons using the Scheffe’s test to determine significant differences between groups. The Kaplan-Meier product-limit method (22) was used to estimate tumor onset functions. The differential effects of SCCA2 protein expression (immunostaining) were assessed using the log-rank test (23), and all of the P-values were related to a two-sided significance test. Statistical procedures were carried out with SPSS 10.1 (SPSS Inc., Chicago, IL) or SAS (SAS Institute, Cary, NC) software.

RESULTS

Identification of Scca2 and Scca2-rs1 Sequences in Car Mice. Microarray results were confirmed by Northern blot analysis, which indicated Scca2 transcript levels to be ~10-fold higher in Car-S than Car-R TPA-treated skin (Fig. 1).

Nucleotide sequence assembly of Scca2 full-length clones obtained by RT-PCR revealed expression of two closely related but distinct Scca2-related sequences in the skin of both Car-R and Car-S mice (Scca2: GenBank accession no. Y144683 and Y144684; and Scca2-rs1: GenBank accession no. Y144685 and Y144476). Car-R and Car-S mice showed no polymorphisms in Scca2 and Scca2-rs1 mRNAs. Alignment of Scca2 and Scca2-rs1 nt or protein sequences revealed high homology (nt identities = 1101/1182 (93%); protein identities = 337/384 (87%)). Car-mice-derived Scca2 and Scca2-rs1 genes were similar but not identical to the reported sequences of mouse Scca2-like genes (NM_009126 and AK00320, respectively).

Absence of LD for Scca2 in Car Mice. Blast analysis of the mouse genome6 with either Scca2 or Scca2-rs1 showed high homology to a cluster of 4 Scca2-related genes mapping from 107.6 to 107.9 Mb on Chromosome 1 (not shown).

Typing of the outbred Car mouse lines (10 mice/group, i.e., 40 chromosomes, equivalent to 720 meioses; Ref. 25) for sixteen microsatellite markers spanning positions 91 to 124 Mb on chromosome 1 revealed no significant LD. Indeed, D1Nds7 (Bc12, D1Mit515, and D1Mit163 markers, mapping proximal to Scca2 at ~107 Mb, and D1Mit137 and D1Mit258 markers, mapping distal to Scca2 at 107.7 and 108.2 Mb, respectively, showed no statistically significant differences in allele frequency in Car-R versus Car-S mice (data not shown).

Effect of Scca2 Overexpression on In Vivo Tumor Growth. Western blot analysis confirmed expression of the transfected Scca2 proteins in stably transfected clones of NCI-H520 human SCC cells (Fig. 2). To determine whether Scca2 might affect tumor growth in vivo, NCI-H520 cells transfected with mouse Scca2, Scca2-rs1, or the

6 Internet address: http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html.
empty cloning vector were injected s.c. into nude mice. Tumor incidence was high in all of the groups (54–90%). As shown in Fig. 3, the tumor growth rates of the Scca2- and Scca2-rs1-transfected groups (n = 10 in each group) were significantly higher than that of the vector-transfected control group (n = 13; \( P < 0.0001 \)). No significant difference in the growth curves was seen between mouse groups injected with Scca2- or Scca2-rs1-transfected cells (Fig. 3). At the end of the observation period (10 weeks from tumor injection) the tumor volumes in the Scca2- and Scca2-rs1-transfected groups were 4–5-fold greater than those of the vector-transfected control group (Fig. 3). These results indicate that Scca2 and the related Scca2-rs1 gene can stimulate \textit{in vivo} growth of NCI-H520 tumor cells.

**SCCA2 Expression in Human SCCs.** Immunohistochemical analysis of 88 skin SCCs from patients of INT (median age at presentation, 62.6 years; range, 36–79 years), revealed SCCA2 antibody reactivity in normal skin of all of the samples (Fig. 4, left), as well as in 65 of 88 (74%) SCCs (Fig. 4, right). However, the remaining 23 tumors showed no immunostaining (Fig. 4, left). Analysis of clinical data indicated a statistically significant association between tumor immunostaining and the age of the patients at tumor onset. Patients with SCCA2-positive tumors were younger (median ± SE = 61 ± 1.2 years) than patients with SCCA2-negative tumor (66 ± 1.3 years; \( P = 0.0018 \), log-rank test; Fig. 5A). A tendency toward a lower histological grade was seen in SCCA2-positive tumors, but the difference was not significant (Table 1), and no significant association between SCCA2 staining and local recurrence or metastases was detected (Table 1). Patients with SCCA2-positive tumors were characterized by a higher frequency of associated basal cell carcinomas (20 of 65 = 31%) as compared with patients with SCCA2-negative tumor (3 of 23 = 13%; Table 1), but the difference was not significant. Eight patients developed additional SCCs at other sites, and 4 patients developed a melanoma, but development did not correlate with SCCA2 expression (data not shown).

A commercial tissue array was used to confirm the association between SCCA2 tumor immunostaining and age of the patient at tumor onset. The array contained 59 Korean samples, including 31 primary SCCs for 88 Italian patients (A) and 30 Korean patients (B) according to SCCA2-positive (light lines) or SCCA2-negative (thick lines) staining. Age at tumor onset was significantly lower in patients whose skin SCCs expressed SCCA2 protein than in SCCA2-negative patients (\( P = 0.0018 \), Italian series; \( P = 0.0158 \), Korean series, log-rank test).
tumors were associated significantly with an earlier clinical presentation ($P = 0.0158$, log-rank test; Fig. 5B).

DISCUSSION

Epidemiological studies suggest that the development of human skin SCC is under polygenic control. This hypothesis is based on the observed excess familial relative risk of skin cancer in offspring of cancer probands (1). Several genetic factors, such as skin type, hair color, and sunburn susceptibility, have been associated with increased risk of skin SCC (2, 3).

Mouse models of polygenic predisposition to cancer are much more amenable to genetic analysis than the corresponding human diseases, where the possible genetic components may be masked by genetic heterogeneity and genetic/environmental interactions.

In the present study, we pursued an approach based on the gene expression profile in the normal tissue of tumor-susceptible and -resistant mice, to identify genes that play a functional role in genetic susceptibility. Our comparison of the gene expression profile between Car-S and Car-R TPA-treated skin revealed overexpression of the Scca2 gene in Car-S mice. The closely related Scca2-rs1 gene was also expressed in skin and detected by the cDNA probe used for Northern blot analysis, which indicated >10-fold higher Scca2/Scca2-rs1 transcript levels in Car-S versus Car-R mice. LD analysis revealed no significant association between line susceptibility and genetic polymorphisms mapping very close to the Scca2 locus. Nucleotide sequence analysis did not identify any Car-line-related polymorphism in the coding region of the Scca2/Scca2-rs1 transcripts. These findings indicate that the Scca2/Scca2-rs1 genes do not show allele-specific effects and suggest that these genes may play a role in skin tumorigenesis as target genes of skin cancer modifier loci. Therefore, we can hypothesize that Car-S-derived cancer susceptibility genes cause a precancerous state in normal cells by deregulating downstream genes (e.g., Scca2) that functionally affect tumor development and growth.

Our transfection experiments demonstrated the direct functional role of Scca2 and Scca2-rs1 in tumor growth. Human SCC NCI-H520 cells transfected with a mammalian expression vector-driving mouse Scca2 or Scca2-rs1 expression grew significantly faster when injected into nude mice than nontransfected or vector-transfected cells, which expressed only low basal levels of Scca2.

The similar effects on tumor growth of the protein products of the Scca2 and Scca2-rs1 genes indicate a similar biochemical function of these two related proteins. The mouse Scca2 protein works as a dual inhibitor of both chymotrypsin-like serine and papain-like cysteine proteinases (26). The Scca2 and Scca2-rs1 mode of action on tumor growth stimulation may involve inhibition of apoptosis, as reported in other systems for the human SCCA2 and SCA1 genes (12, 13, 27). Our findings are consistent with the recent observation that growth of SKG IIIa tumor cells transduced with a recombinant retrovirus expressing antisense SCCA is inhibited significantly (13).

Early age at onset of a tumor is characteristic of a genetic predisposition to cancer, as observed in several human familial cancer syndromes, including breast cancer predisposition resulting from BRCA1 gene mutations and colorectal cancer in Lynch syndrome patients (28, 29). Our immunohistochemical analysis of SCCA2 protein expression in human skin SCC, using a monoclonal antibody that specifically detects SCCA2 protein (21), revealed a significant association between SCCA2 protein expression and early age at tumor presentation in both the INT samples and the commercial tissue array of skin SCCs from Korean patients.

In conclusion, our study demonstrating elevated Scca2 expression in the TPA-treated skin of Car-S mice and in the skin SCCs of patients with early tumor onset, provides evidence for a role of Scca2 gene expression in genetic predisposition to skin cancer in a murine model as well as in humans.

ACKNOWLEDGMENTS

We thank Barbara Vergani, Maria Carmen Intronti, Desirè Parimbelli, and Carmen Pignatiello for technical assistance.

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