Aberrant Expression of Collagen Triple Helix Repeat Containing 1 in Human Solid Cancers

Liren Tang,1,2 Derek L. Dai,1 Mingwan Su,1,2 Magdalena Martinka,3 Gang Li,1 and Youwen Zhou1,2,4

Abstract

Purpose: The collagen triple helix repeat containing 1 (CTHRC1) is a promigratory protein first found to be expressed during rat tissue repair process. Recent preliminary results revealed CTHRC1 mRNA in melanoma and breast cancer. However, the full significance of CTHRC1 to human carcinogenesis remains unclear. This study is to further characterize the clinical and functional relevance of CTHRC1 in melanoma and other human solid cancers.

Experimental Design: First, semiquantitative immunohistochemistry analysis was done on 304 clinically annotated, paraffin-embedded biopsies representing different stages of melanoma progression. Then, short interfering RNA was used to inhibit expression of CTHRC1 protein for migration analysis on cultured melanoma cells. Finally, the CTHRC1 expression was surveyed in 310 samples representing 19 types of human solid cancers.

Results: In benign nevi and noninvasive melanoma biopsies, there was little CTHRC1 protein expression. In contrast, in invasive primary melanomas, there was a significant increase of CTHRC1 protein ($P < 0.01$,$\chi^2$ test). There was a further increase of CTHRC1 protein in metastatic melanoma specimens compared with nonmetastatic lesions ($P < 0.01$,$\chi^2$ test). In addition, inhibition of CTHRC1 expression resulted in decreased cell migration in vitro. Finally, transcription survey in 19 types of human solid cancers revealed aberrant CTHRC1 expression in 16 cancer types, especially cancers of the gastrointestinal tract, lung, breast, thyroid, ovarian, cervix, liver, and the pancreas.

Conclusions: Aberrant expression of CTHRC1 is widely present in human solid cancers and seems to be associated with cancer tissue invasion and metastasis. It potentially plays important functional roles in cancer progression, perhaps by increasing cancer cell migration.

Materials and Methods

Melanoma and nevi biopsies and RNA preparation. Metastatic melanoma nodules and benign nevi were obtained from patients...
with metastatic melanoma, with informed consent as approved by the University of British Columbia Clinical Ethics Board (Vancouver, British Columbia, Canada). The biopsies were collected as described previously (12). Briefly, biopsies were placed in the centers of the lesions and each lesion was sectioned into two equal parts. One part was formalin fixed and examined histologically for diagnostic confirmation and for estimation of melanoma/melanocyte content (at least 80%). The other part was immediately frozen in liquid nitrogen. The freshly frozen specimens were extracted for total RNA using Trizol protocol (In vitrogen, Burlington, Ontario, Canada) according to the manufacturer's instructions. The formalin-fixed, paraffin-embedded archival biopsies used for immunohistochemistry were obtained from the archival collection of the Department of Pathology, Vancouver General Hospital (Vancouver, British Columbia, Canada).

Quantitative PCR. The cDNA synthesis reaction was done using 1 μg total RNA for each sample using cDNA SuperScript First-Strand Synthesis System (Invitrogen). A portion of the resultant cDNA equivalent to 4 ng total RNA was used for quantitative real-time reverse transcription-PCR using the DNA Engine Opticon System (MI Research, Waltham, MA). The primer sequences for the genes tested include the following: CTHRC1, 5'-TCACTGACTCTTCCTTGTTG-3' (forward) and 5'-GCCAACCCGATAGAACATG-3' (reverse); S-100b, 5'-ATCC-GAATCGAGGCTCATC-3' (forward) and 5'-CTGGTCTACATCGTGATGT-3' (reverse); tyrosinase, 5'-TCCTTTGATCTTCAGGATT-3' (forward) and 5'-TTCGACGTTGACTGTAAGG-3' (reverse); tyrosinase-related protein 1, 5'-GCAATGTTGTACGACCGG-3' (forward) and 5'-AAGTCTGACGCTGCTTC-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-AACATCATCGAGATTGTC-3' (forward) and 5'-TGGACTGTGGTCATGCCT-3' (reverse). The PCR reactions were performed in triplicate in a volume of 20 μL using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The amplification conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cycle number of threshold (C_T) was recorded for each reaction. The C_T value of CTHRC1 was normalized to that of glyceraldehyde-3-phosphate dehydrogenase of the same sample. The expression intensity of each gene in metastatic melanoma was expressed as fold changes over the average of nevus samples.

Antibody production and Western blotting. A polyclonal antibody was generated in the laboratory by immunizing two rabbits with a synthetic peptide (NH_2-CRSIIHEELPK-COOH) from the COOH terminal of CTHRC1 protein. The immune sera were affinity purified according to the manufacturer's instructions. The antibody against the COOH terminal of the CTHRC1 protein and horseradish peroxidase conjugates (LSAB+System, horseradish peroxidase) were purchased from DAKO. The visualization of the staining was achieved using DAKO Liquid 3,3′-Diaminobenzidine Substrate-Chromogen System. Normal rabbit serum at the same concentration was used on adjacent tissue sections as negative control.

Construction and clinical annotation of melanoma tissue microarrays were described previously (12, 23). The current melanoma tissue microarray consists of 16 human normal nevi, 204 primary melanomas, and 58 metastatic melanomas. Due to loss of biopsy cores or insufficient tumor cells present in the cores, 10 cases of normal nevi, 154 cases of primary melanomas, and 54 cases of melanoma metastases could be evaluated for CTHRC1 staining. Combined with individual sections, a total of 304 samples were evaluated by immunohistochemistry.

To quantify the CTHRC1 staining intensity, a three-point scoring scale was used: -1, no cells or ≤10% of cells stained; +2, majority of cells positive and moderately stained; and +3, >100% cells positive and strongly stained (staining equivalent to or greater than that seen with S-100 antibody). Three independent investigators, including a dermatopathologist (M.M.), who were blinded of the clinical diagnosis of the sections and tumor thickness, assessed the stained slides, and a consensus score was reached for each sample. Because the staining results from individual sections were similar to that from the tissue microarray sections, the samples were combined in the final analysis. The χ² test was used to evaluate the statistical differences between the staining of different stages of melanocytic tumors. The same method was used to evaluate potential correlation between CTHRC1 protein expression in primary melanoma samples and the clinical characteristic of the melanoma patients, such as tumor thickness and survival.

Normal melanocytes and melanoma cell lines. Two normal human epidermal melanocyte cell lines were isolated in the laboratory from neonatal foreskins as described (24), and one normal melanocyte cell line was purchased from Cascade BioScience, Inc. (Portland, OR). The melanocytes were cultured with melanocyte growth medium (Cascade BioScience) and maintained at 37°C with 5% CO_2. Cultures were provided with fresh medium thrice weekly, and melanocytes were used at passage 4 for RNA and protein extraction.

The melanoma cell lines were described previously (12). Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. All the cultures were replenished with fresh medium 24 hours before harvesting for protein and RNA extraction.

RNA interference. The target short interfering RNA (siRNA) sequences of CTHRC1 are as follows: 5'-CCCCATGAGCTAATTATTT-3' (target 1) and 5'-CCGCACTCATTTGAAGACTA-3' (target 2). The RNA duplexes were synthesized by Qiagen (Mississauga, Ontario, Canada). The control siRNA was purchased from Ambion (Houton, TX) as a 50 μmol/L stock, and it is a RNA duplex synthesized from a random sequence. For siRNA transfection, melanoma cells were seeded 24 hours before the transfection in antibiotics-free DMEM containing 10% fetal bovine serum. The cells were transfected with 50 nmol/L siRNA using LipofectAMINE (Invitrogen) for 6 hours in antibiotics-free medium. Then, the medium was replaced with the normal DMEM with 10% fetal bovine serum containing proper antibiotics.

Cell migration assay. For migration assays, the siRNA-transfected cells after 24 hours were trypsinized and seeded onto top of the insert membrane (0.8 μm) of the Boyden chamber (BD Biosciences, Palo Alto, CA) in 0.1% fetal bovine serum in DMEM on a 24-well tissue culture
CTHRC1 expression in cancer cDNA arrays. A PCR-generated CTHRC1 cDNA fragment (forward primer, 5'-GCCAATGGCATCC-GGCTAGC-3'; reverse primer, 5'-TGTGAATCTACACTTTGGTCTG-3') was $^{32}$P labeled using the ready-to-go DNA-labeling beads (Amersham Biosciences). A membrane containing 155 pairs of tumors and their matching normal control cDNAs (Cancer Profiling Array II, BD Biosciences) was probed with the labeled CTHRC1 fragment at 68°C according to the manufacturer’s instruction. The membrane was exposed to the film at −70°C, and the film was developed after 12 hours. A cDNA fragment of the control housekeeping gene ubiquitin provided by BD Biosciences was rehybridized to the stripped membrane and developed after 1-hour exposure. The signal intensity for each tumor-normal tissue pair was obtained using GenePix Pro 4.1 software (Axon Instrument, Sunnyvale, CA) for analyzing autoradiography images of CTHRC1 hybridization. The CTHRC1 signal intensity was normalized to that of ubiquitin of the same sample. The ratio of tumor over normal control tissues was obtained from each paired sample.

Results

Identification of a novel cancer-associated gene, CTHRC1, in melanoma. In an attempt to explore differentially expressed genes between normal benign nevi and metastatic melanomas, we did DNA microarray analysis representing >21,000 genes comparing the normal nevi and metastatic melanoma nodules from the same patients (12). Several genes were shown to be overexpressed in metastatic melanocytes compared with the benign melanocytes, including osteopontin, which subsequently was found to be associated with melanoma invasion (12). Another melanoma differentially expressed gene identified is CTHRC1. This gene was highly overexpressed in metastatic melanomas but barely detectable in normal nevi. Quantitative reverse transcription-PCR analysis confirmed the elevated expression of CTHRC1 in metastatic melanoma specimens (n = 7) when compared with normal nevi samples from 14 patients (Fig. 1). On average, metastatic specimens exhibited eight times more expression of CTHRC1 mRNA compared with the normal nevi of similar melanocyte content (~80% for both metastatic melanoma nodules and benign i.d. nevi). The melanocytic marker S-100 was similarly present in the normal nevi and metastatic melanoma nodules, confirming that the differential expression of CTHRC1 was not due to unequal melanocyte content. In addition, the metastatic melanoma nodules expressed less melanocyte differentiation markers, such as tyrosinase and tyrosinase-related protein 1, which frequently are lost during melanoma progression (Fig. 1; ref. 25).

On Western blotting, no CTHRC1 expression was detected from three melanocyte cultures generated from different sources, whereas CTHRC1 was easily detected from melanoma cell lines (Fig. 2). A major polypeptide band of ~25 kDa on Western blots was detected. This corresponds to the expected size of the human CTHRC1 and was verified by transfecting expression vectors, directing the synthesis of CTHRC1 into cultured mammalian cells (data not shown).

CTHRC1 protein expression is correlated with melanoma invasion and metastasis. Using immunohistochemistry staining, the CTHRC1 protein was in melanoma cells of primary and metastatic melanoma biopsies. In contrast, no significant expression was present in benign nevi or noninvasive melanoma in situ cells (Fig. 3). The specificity of CTHRC1 antibody staining of melanocytic cells in immunohistochemistry was shown using S-100 antibody on adjacent sections. Using a three-point staining intensity scale, the relative expression of CTHRC1 protein in a spectrum of melanocytic tumors was quantified, combining results from 304 clinical specimens (Fig. 3B). The CTHRC1 protein expression was weak or absent in benign nevi (normal nevi) and in non-invasive melanocytic tumors (melanoma in situ). In contrast, in melanoma samples that have invaded into the dermis (invasive primary melanomas), a dramatic increase of CTHRC1 expression was present, with ~70% of tumors showing moderate to strong expression. The difference between benign nevi, noninvasive melanoma, and invasive primary melanoma was highly significant (P < 0.001, $\chi^2$ test). Further increase of CTHRC1 protein expression was observed in samples of metastatic melanoma, with >80% samples stained moderately to strongly. The difference between primary invasive melanoma and metastatic melanoma is also statistically significant (P < 0.01, $\chi^2$ test).

Because the expression in primary melanoma was nonuniform, an attempt was made to correlate with patient’s clinical characteristics, such as tumor thickness, 5-year disease-free survival, and overall survival. However, no significant correlation was identified (data not shown).

CTHRC1 promotes melanoma cell migration. To examine the functional roles of CTHRC1 overexpression in melanoma cells, cultured KZ-28 melanoma cells were transfected with two siRNA duplexes against CTHRC1. As shown in Fig. 4A and B, both CTHRC1 siRNA oligos effectively blocked CTHRC1 expression at both mRNA and protein levels in KZ-28 cells.
Similar results were observed in another metastatic melanoma cell line, MMRU (data not shown). Because rat fibroblasts overexpressing CTHRC1 enhanced cell migration (17), we hypothesized that CTHRC1 will do the same for melanoma cells. The effect of CTHRC1 reduction on the migratory behaviors of melanoma cells was tested using Boyden chamber. At 24 hours, there was a 50% reduction of cell migration in the CTHRC1 “knockdown” cells compared with cells transfected with the control, nonspecific siRNA. At 48 hours, the reduction of migration by the CTHRC1 knockdown cells was further enhanced (Fig. 4C). Both CTHRC1 siRNA sequences had similar effects. The CTHRC1 siRNA2 was used in Fig. 4.

**CTHRC1 expression is aberrantly up-regulated in the majority of human solid cancers.** There is little information on the expression of this newly characterized human gene in human cancers. The only reports to date were on the transcript expression of CTHRC1 in breast cancer stromal cells using DNA microarrays and in situ hybridization (21, 22). To explore the expression of CTHRC1 in other tumor types, we used a CTHRC1-specific gene probe to hybridize to a membrane that contained 310 cDNA samples derived from clinical tumor biopsies from 155 cancer patients, each with one tumor biopsy and one match normal tissue (Cancer Profiling Array II). Nineteen types of human solid cancers were represented on this membrane. CTHRC1 expression was dramatically and aberrantly up-regulated in the vast majority of human cancers compared with the corresponding normal tissues (Fig. 5A). Especially, in the cancers of rectum, small intestine, colon, liver, lung, ovary, breast, thyroid gland, and cervix, almost all the tumor samples expressed more CTHRC1 mRNA compared with their normal control samples (Fig. 5A and B). Among the 10 skin cancers and their normal controls included on the membrane, 5 of 7 melanomas expressed more CTHRC1 mRNA than normal skin, whereas the 3 squamous cell carcinomas expressed less CTHRC1 than normal skin. The tumors of testis, prostate, and trachea showed no significant CTHRC1 expression changes compared with their normal controls.

**Discussion**

The current study describes the aberrant expression of the novel cancer-associated gene CTHRC1 in melanoma and many other human solid cancers and investigated the potential roles played by CTHRC1 in cancer cells. There is little information previously known about this gene in human cells. The overall domain structure is the same as that of rat and mouse, containing an NH2-terminal signal peptide for extracellular secretion, a short collagen triple helix repeat of 36 amino acids, and a COOH-terminal globular domain (17). There is a 92% amino acid sequence identity between human and rat CTHRC1 proteins (data not shown).

The possible involvement of CTHRC1 in human carcinogenesis was first suggested by a recent report that some stromal cells in breast cancer expressed CTHRC1 mRNA by cDNA microarray analysis and in situ hybridization (21). The results presented here suggest that melanoma cells aberrantly express both CTHRC1 mRNA and CTHRC1 protein (Figs. 1-3). Although it cannot be excluded that stromal cells in melanoma
tissues also express this protein, the main source of CTHRC1 protein in melanoma tissues is, however, likely to be melanoma cells because the staining was mainly in S-100 staining cells of the tissue sections (Fig. 3) and that pure cultures of melanoma cell lines expressed this protein using Western blotting analysis (Fig. 2).

The functional significance of CTHRC1 expression in melanoma progression is unknown. The observation that it is absent in noninvasive stages of melanoma (melanoma in situ) and greatly increased in samples of invasive primary melanoma suggests that it may be important in tumor cell invasion into the dermal tissue. In addition, a further statistically significant increase was observed in metastatic melanoma, suggesting a possible role at this critical step of melanoma progression as well. Further experiments are required to verify these associations.

The exact molecular role played by CTHRC1 in melanoma cells is also unknown. However, because in rat cells, the increased expression caused the fibroblast cells to have increased migration (17), it might be possible that CTHRC1 has a promigratory role in melanoma cells. Indeed, when CTHRC1 in melanoma cells was inhibited by siRNA, there was a significant reduction in cell migration (Fig. 4). Because tissue invasion and metastasis both involve cancer cell migration, it is possible that CTHRC1 contributes to these steps by increasing tumor cell migration.

At present, the mechanism of CTHRC1 aberrant expression in metastatic melanoma and the molecular mechanisms of CTHRC1 promigration function are not clear. In murine NIH3T3 cells, CTHRC1 was induced by transforming growth factor-β and bone morphogenetic protein-4 (17). Melanoma cells produce transforming growth factor-β and bone morphogenetic protein-4, and expression of both directly correlates with the advanced depth of tumor invasion (26–30). Sequence analysis of the CTHRC1 promoter region reveals a binding site of SMAD, which is responsive to transforming growth factor-β/bone morphogenetic protein regulation. Therefore, it is possible that up-regulation of CTHRC1 in melanoma cells is due to activation of transforming growth factor-β/bone morphogenetic protein-4 pathway.

It is not clear about how CTHRC1 regulates melanoma cell migration. Like osteopontin, another secreted extracellular protein found to be overexpressed by melanoma cells from our laboratory (12), CTHRC1 may regulate cell migration as a bridging and/or cell adhesion protein. Consistent with this, CTHRC1 and osteopontin have been found to be colocalized in calcified atherosclerotic plaque particularly at the calcifying front (17, 31). To date, a direct interaction between CTHRC1 and osteopontin has not been studied. It is also possible that CTHRC1 produced by melanoma cells promotes tumor invasion by regulating the surrounding microenvironment, most likely the stromal cells and extracellular matrix. Numerous reports showed that the tumor microenvironment is pivotal for cancer cell growth/survival, invasion, and metastasis (32–34). Crucial stromal cells in the environment are fibroblasts (also termed cancer-associated fibroblasts) that are located in the vicinity of the neoplastic cells. We have detected CTHRC1 protein from conditioned medium of cultured melanoma cells.5 Because mouse fibroblasts almost completely shut down collagen I gene transcription in the presence of increased CTHRC1 protein (17), it is possible that the secreted form of human CTHRC1 overproduced by melanoma cells acts on stromal fibroblasts to decrease synthesis of extracellular matrix components, such as collagen I, and thus prepares the extracellular environment for tumor invasion, survival, and metastasis.

Human tumor cDNA array analysis showed that, with the exception of testis and prostate cancers, the CTHRC1 aberrant expression seems to be widely present in solid human cancers. Because CTHRC1 seems to regulate melanoma cell migration, it may also be important for most of the other solid cancers surveyed in this study.

Fig. 4. Knockdown of CTHRC1 expression by siRNA reduced melanoma cell migration. A, inhibition of CTHRC1 mRNA expression by siRNA in KZ-28 melanoma cells. Cultured KZ-28 cells were treated with synthetic control or two CTHRC1-specific siRNA oligos for 24 hours, and the cells were harvested for RNA extraction and for quantitative PCR analysis with primers designed from CTHRC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Both siRNAs efficiently blocked CTHRC1 mRNA expression, whereas the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA did not change. B, inhibition of CTHRC1 protein expression by siRNA in KZ-28 melanoma cells. Cultured KZ-28 cells were treated the same way as above and harvested for Western blotting with anti-CTHRC1 antibody. Specific and efficient inhibition was achieved using both CTHRC1-specific siRNA oligos. β-Actin levels were not altered. C, reduction of migrated melanoma cells by CTHRC1 siRNA. siRNA-transfected KZ-28 cells were seeded on top of Boyden chamber. The cells that migrated to the other side of the membrane were counted at 24 and 48 hours. The average number of cells migrated was compared between the control and CTHRC1 siRNA-transfected cells. Significant differences between control siRNA-transfected and CTHRC1 siRNA2-transfected cells were observed (P < 0.01, Student’s t test).

5 Unpublished data.
In summary, we have found that CTHRC1 aberrant up-regulation is present in the vast majority of human cancers. In addition, this up-regulation seems to be correlated with cancer progression steps, such as tumor invasion and metastasis. CTHRC1 may be functionally important in these steps by increasing tumor cell migration. Targeting CTHRC1 may represent an attractive approach for developing a wide-spectrum cancer therapy for a variety of human cancers.

Fig. 5. CTHRC1 expression in human solid cancers. The CTHRC1 cDNA fragment was hybridized to Cancer Profiling Array II, representing 19 human cancers and 10 cancer cell lines. Each tumor sample (T) is matched with its own normal control tissue (N). The same array was stripped and reprobed with the housekeeping ubiquitin gene fragment. A, image probed with CTHRC1 fragment. B, image of ubiquitin expression on the same membrane.

References
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