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Mutations in BALB mitochondrial DNA induce CCL20 up-regulation promoting tumorigenic phenotypes

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ABSTRACT

mtDNA mutations are common in human cancers and are thought to contribute to the process of neoplasia. We examined the role of mtDNA mutations in skin cancer by generating fibroblast cybrids harboring a mutation in the gene encoding the mitochondrial tRNA for arginine. This somatic mutation (9821insA) was previously reported in UV-induced hyperkeratotic skin tumors in hairless mice and confers specific tumorigenic phenotypes to mutant cybrids. Microarray analysis revealed and RT-PCR along with Western blot analysis confirmed the up-regulation of CCL20 and its receptor CCR6 in mtBALB haplotype containing the mt-Tr 9821insA allele compared to wild type mtB6 haplotype. Based on reported role of CCL20 in cancer progression we examined whether the hyper-proliferation and enhanced motility of mtBALB haplotype would be associated with CCL20 levels. Treatment of both genotypes with recombinant CCL20 (rmCCL20) resulted in enhanced growth and motility of mtB6 cybrids. Furthermore, the acquired somatic alteration increased the *in vivo* tumor growth of mtBALB cybrids through the up-regulation of CCL20 since neutralizing antibody significantly decreased *in vivo* tumor growth of these cells; and tumors from anti-CCL20 treated mice injected with mtBALB cybrids showed significantly decreased CCL20 levels. When rmCCL20 or mtBALB cybrids were used as chemotactic stimuli, mtB6 cybrids showed increased motility while anti-CCL20 antibody decreased the migration and *in vivo* tumor growth of mtBALB cybrids. Moreover, the inhibitors of MAPK signaling and NF-κB activation inhibited CCL20 expression in mtBALB cybrids and decreased their migratory capabilities. Thus, acquired mtDNA mutations may promote tumorigenic phenotypes through up-regulation of chemokine CCL20.

1. Introduction

Tumor development and progression are multifactorial processes with complex regulation. The members of chemokine superfamily are considered to be important elements that can regulate neoplastic processes in cancer cells. Chemokines and their receptors are expressed by tumor and/or by host cells, in primary tumors, and in specific metastatic loci. Some of them support tumor development and progression mostly by their ability to induce cellular motility while the others can potentially suppress cellular functions that are involved in malignant transformation [1]. Generally, chemokines can play an important role in formation of primary tumors and metastases [2].

An important member of chemokine superfamily is chemokine CCL20. CCL20 was identified in 1997 by three independent groups in screens of human cDNA libraries from liver, monocytes and pancreatic cells and was designated liver and activation-regulated chemokine (LARC) [3], macrophage inflammatory protein-3α (MIP-3α) [4], and Exodus-1 [5], respectively. Thus, in the systematic chemokine nomenclature, LARC/MIP-3α/Exodus-1 is designated as CCL20 (CC chemokine ligand 20) [6]. CCL20 can function as both an inflammatory and a homeostatic chemokine depending on the specific situation and its natural receptor is the CCR6. Their interaction regulates multiple physiological functions, particularly tissue

Abbreviations: mtDNA, mitochondrial DNA; UV, ultraviolet; CCL20, CC chemokine ligand 20; rmCCL20, recombinant protein CCL20; LARC, liver and activation-regulated chemokine; MIP-3α, macrophage inflammatory protein-3α; CCR6, CC chemokine receptor type 6; NF-κB, nuclear factor-κB; IκB, inhibitor of kappa B; ROS, reactive oxidative species; NMSC, non-melanoma skin cancer; MMP, metalloproteinases; Col1A1, collagen, type I, alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; NAC, *N*-acetyl cysteine; tBHP, tert-butyl hydroperoxide; RT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction.

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architecture and compartment-specific migration of white blood cells [7]. Cancer cells can also exploit the CCL20/CCR6 receptor system for mediation of their specific migration and metastasis [8]. It was observed that CCL20 as well as CCR6 play important role in colorectal cancer leading to enhanced proliferation and migration. Compared to normal colon mucosa, CCR6 and CCL20 both were found to be up-regulated in colorectal cancer and colorectal liver metastasis [9]. CCL20 participation in cancer progression was also shown in pancreatic adenocarcinoma where CCL20 expression was significantly higher compared to normal tissue [10–12]. Huang and Geng [13] made similar observation in hepatocellular carcinoma samples where significantly enhanced expression of both CCL20 and CCR6 was seen compared to healthy tissue. CCL20 was also shown to be up-regulated in biopsies of breast cancer patients [14,15], renal cell carcinoma [16], melanoma [17] and squamous cell carcinoma including keratinocytes [18]. Baumforth et al. [19] observed up-regulation of CCL20 caused enhanced migration of regulatory T cells in Hodgkin's lymphoma patients.

Expression of chemokine ligand CCL20 is controlled by nuclear factor-κB (NF-κB) transcription factor [20]. NF-κB plays an important role during cellular responses to inflammatory stimuli and general responses to pathogens in a number of different cell types and is inhibited by the IκB molecule. IκB phosphorylation and its subsequent degradation releases NF-κB triggering transcription of many nuclear genes involved in pro-carcinoma processes, including chemokine CCL20 and targeting NF-κB by its specific inhibitors results in suppression of CCL20 expression in cells [21]. Besides of the NF-κB-dependent CCL20 expression, it is known that the promoter region of CCL20 contains binding sites for the Ets transcription factor which is activated by ERK1/2 suggesting a role of the Ras-MAPK-pathway in CCL20 expression [22].

In our study, we employed a cybrid model for analysis of mtDNA changes found in UV-induced non-melanoma skin cancer (NMSC). We previously discovered a mutation hot spot (9821insA) in mouse *mt-Tr* locus ($tRNA^{Arg}$) in approximately one third of premalignant and malignant skin tumors (squamous papillomas and squamous cell carcinomas). To determine the functional relevance of this particular mutation *in vitro*, cybrid cells with the same nuclear background but containing different *mt-Tr* ($tRNA^{Arg}$) alleles were generated [23]. We demonstrated that a 9821insA can alter the biochemical characteristics of murine cybrids and subsequently can contribute to significant changes in their behavioral capabilities. Moreover, we showed that there are mtDNA-driven differences in ROS production between wild type (mtB6) and 9821insA (mtBALB) cybrids [23,24]. Microarray analysis was conducted in order to elucidate the expression differences between the two cybrid lines differing only in their mtDNA. 44 K mouse whole genome chip revealed roughly 400 differentially expressed genes between the two cybrid lines including genes involved in tumorigenic processes such as matrix matalloproteinases (MMPs) and inflammatory chemokines and their receptors [25].

Chemokine CCL20 and its receptor CCR6 were found up-regulated in mutant cybrids. Based on previously reported role of CCL20/CCR6 in tumor progression we examined whether CCL20/CCR6 is also responsible for the mtBALB haplotype oncogenic features like enhanced proliferation, migration and invasion. Targeting expression of CCL20 by its natural upstream transcription factor NF-κB and Ets; or disruption of CCL20/CCR6 interaction could be a useful strategy in a treatment of cancer. When combined with other therapeutic approaches, the research on chemokines and namely CCL20 in various cancers may provide novel means for improved outcomes.

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The IACUC protocol was approved by the University of Arizona Institutional Animal Care and Use Committee (Permit number 07-029). All procedures were performed in every effort to minimize suffering.

2.2. Cybrid cell lines

LMEB3(mtBALB) and LMEB3(mtB6) cells were generated by harvesting the mitochondria from brain synaptosomes of B6 and BALB mice and electrofusing them to a mouse fibroblast LMEB3ρ^0 cell line that lacked its own mtDNA. Two cybrid lines differing only in their mtDNA but having identical nuclear background from murine LA9 cells were produced. A mouse LMEB3ρ^0 cell line was produced by exposure of LM(TK-) cells to ethidium bromide as described previously by [26]. Both lines were maintained in DMEM medium supplemented with 10% FBS and Pen/Strep (Life Technologies, Grand Island, NY) in 5% CO_2 atmosphere at 37 °C.

2.3. DNA sequencing of mitochondrially encoded $tRNA^{Arg}$ (mt-Tr locus) in cybrid lines

Forward (5′-TTCTAGTCACAATTCTATCTCTAGGC-3′) and reverse (5′-GCATTGTAGTAGGTTGAGATTTTGG-3′) primers were designed for PCR to amplify the *mt-Tr* gene from total genomic DNA of both cybrid lines. PCR samples were prepared in a total volume of 50 μl. Each tube contained 1xTaq master Mix (New England BioLabs) containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 25 units/ml Taq DNA polymerase, 5 mM of both primers, 0.2 mM dNTPs each, 5% glycerol, 0.08% NP-40, and 0.05% Tween-20 and 250 ng of DNA template of either mtB6 or mtBALB cybrid cells. The PCR parameters were as follows: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, and elongation at 68 °C for 1 min, with a final elongation at 68 °C for 5 min. PCR products were then purified using the QIAquick PCR purification kit (Qiagen) and sequenced on an Applied Biosystems 3730XL DNA analyzer (Life Technologies, Carlsbad, CA).

2.4. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

mRNA expression analysis was performed as described previously [25] with further modifications. Mouse CCL20 (Mm00444228_m1), CCR6 (Mm99999114_s1) and GAPDH (Mm99999915_m1) primer/probes purchased from ABI (Applied Biosystems, Branchburg, NJ) were used. PCR amplification of GAPDH was used to control the quality of cDNA. CCL20 and CCR6 levels were normalized to GAPDH control. Amplification plots were generated and the Ct values (cycle number at which fluorescence reaches threshold) recorded.

In experiments with rmCCL20, the cybrids were incubated 24 h with 150 ng/ml rmCCL20 (R&D Systems, Minneapolis, MN) and then allowed growing for an additional 24 h in culture. Thereafter, cells were harvested and RNA isolated.

In experiments with inhibitors PD98059 or BAY11-7082, cybrids were pre-treated with 50 μM PD98059 (Sigma-Aldrich, St. Louis, MO) and/or 5 μM BAY11-7082 (EMD Chemicals, Gibbstown, NJ) dissolved in DMSO, or vehicle alone for 60 min in serum free medium. After the treatment, medium was replaced with the normal growing medium (supplemented with 10% FBS) and the same