

ELECTRONIC LETTER

Significant involvement of CCR2-64I and CXCL12-3a in the development of sporadic breast cancer

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The molecular biology of cancer is still far from being understood, with the exception of specific familial cases. Amplifications of oncogenes and alterations in tumour suppressor and detoxification genes by mutations or deletions appear especially important in the development of sporadic breast tumours.¹⁻³

Tumour infiltrating lymphocytes (TILs) and tumour associated macrophages are thought to play a crucial role in tumour immune surveillance and possibly development. The activation and recruitment of lymphocytes is regulated by chemotactic and proinflammatory chemokines such as RANTES (CCL5), MCP-1, and to a lesser extent MIP-1 alpha, MIP-1 beta, and IL-8.⁴⁻⁷ It has been suggested that melanoma cells evade immune surveillance through the induction of TIL cell death by SDF-1 alpha (CXCL12) and RANTES.⁸ MCP-1 is the natural ligand of the CCR2 chemokine receptor, expressed mainly in the monocytes, activated T lymphocytes, and memory cells,⁹ whereas RANTES is the ligand of CCR5.¹⁰

The relevance of chemokines to malignancy extends beyond leucocyte recruitment. Animal models have shown that chemokine secretion by tumour cells can influence angiogenesis and tumour growth. Expression of angiogenic CXC chemokines by tumour cells in severe combined immunodeficient (SCID) syngenic mice has been shown to enhance tumour growth.¹¹ However, the association of any kind of cancer with chemokine related genetic markers has not been examined to date.

We selected the polymorphisms CCR2-64I,¹² CXCL12-3'A, CCR5Δ32, and CCR5 59029 G-A¹⁰ because of functional and clinical data from AIDS studies. We determined the genotype for the above four polymorphisms in 442 cancer samples and 361 control samples. Our data indicate a significant involvement of the chemokine system in the development of breast cancer.

MATERIALS AND METHODS

Patients and samples

Breast cancer

Blood samples were collected at the Prolepsis (centre for breast cancer diagnosis and research in Athens, Greece) from 233 female patients with breast cancer, aged 31-79 (average 56) years. Samples were stored at 4°C until DNA extraction. In addition, 31 tumour specimens from female breast cancer patients, aged 36-76 (average 56) years, were surgically obtained and stored at -70°C until DNA extraction.

Non-melanoma skin cancer

Blood samples were collected at the A Sygros Hospital (Athens, Greece) from 110 non-melanoma skin cancer patients (23 with squamous cell carcinoma, five with Bowen's disease, 72 with basal cell carcinoma, and 10 with other premalignant lesions). The age of the subjects ranged from 39 to 90 (average 68) years; 50 were men and 60 were women. The specimens were stored at 4°C until DNA

Key points

- The CCR2-64I allele conferred significant protection from breast cancer.
- The mutated CXCL12 allele conferred increased susceptibility to breast cancer.
- Polymorphisms in the CCR5 gene (Δ32, 59029 G-A) showed no association with breast cancer.
- No association was found in the bladder and skin cancer groups with any of the chemokine polymorphism studied.

extraction. The diagnosis in all cases was histologically confirmed.

Bladder cancer

At the Department of Urology, University General Hospital of Heraklion, Greece, 68 tumour specimens were obtained from male patients aged 47-81 (average 66.6) years with histologically confirmed bladder cancer. The specimens were stored at -80°C immediately after surgical removal, until DNA extraction.

Control groups

Blood samples were collected from 361 healthy donors aged 19-82 (average 49.1) years registered with a general practice in Athens, Greece. For the female breast cancer patients, 210 women aged 25-82 (average 53.5) years formed a control group; and for the male bladder cancer patients, 148 men aged 19-76 (average 44) years were controls. The total healthy population of 361 donors (58% women) formed a control group for the skin cancer patients (54% of these were women).

All subjects derived from a white Caucasian Greek population. All the cancer patients in the study had no family history of cancer and presented tumours which were characterised as sporadic primary. The Ethics Committee of the University of Crete approved this study and written informed consent was obtained from all donors. Genotyping of tumour suppressor genes from cancer tissues includes the risk of introducing artefacts because of the inherent genomic instability. Although none of the genes examined in the present study fell into the tumour suppressor gene category, to reduce even further the possibility of artefacts, in the cases where a blood sample was lacking the adjacent normal portion of the excised cancer tissue was used.

DNA extraction and genotyping

DNA extraction was carried out according to previously published methods.¹⁴ All specimens were examined for the presence of amplifiable DNA. In each PCR, two negative

controls were employed to make sure that no contaminants were introduced in the initial PCR. The primers used in the study were:

- CCR5 Δ 32F: 5'-GTGGTGACAAGTGTGATCAC-3'
- CCR5 Δ 32R: 5'-TTGTAGGGAGCCCAGAAGAG-3'
- CXCL12F: 5'-CAGTCAACCTGGGCAAAGCC-3'
- CXCL12R: 5'-AGCTTTGGTCTGAGAGTCC-3'
- CCR2F: 5'-TTGTGGGCAACATGATGG-3'
- CCR2R: 5'-CTGTGAATAATTTGCACATTGC-3'
- CCR5_59029F: 5'-CCCGTGAGCCCATAGTAAAACTC-3'
- CCR5_59029R: 5'-TCACAGGGCTTTCAACAGTAAGG-3'.

The reaction mixture consisted of PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 mM of each primer, and 1.2 units/reaction Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The reaction conditions were:

- for CCR5 Δ 32, an initial denaturation at 94°C for three minutes, then 35 cycles at 92°C for 30 seconds, at 55°C for 30 seconds, at 72°C for 30 seconds, and finally extension at 72°C for 10 minutes¹⁵
- for CXCL12-3A, PCR conditions as above, with annealing temperature at 58°C; PCR products were digested with 10 units MspI/reaction (NEBS) at 37°C for four hours¹⁶
- for CCR2-64I 3A, PCR conditions as above, with annealing temperature at 52°C. The products were digested with 10 units BSA BI /reaction (NEBS) at 60°C for four hours¹⁷

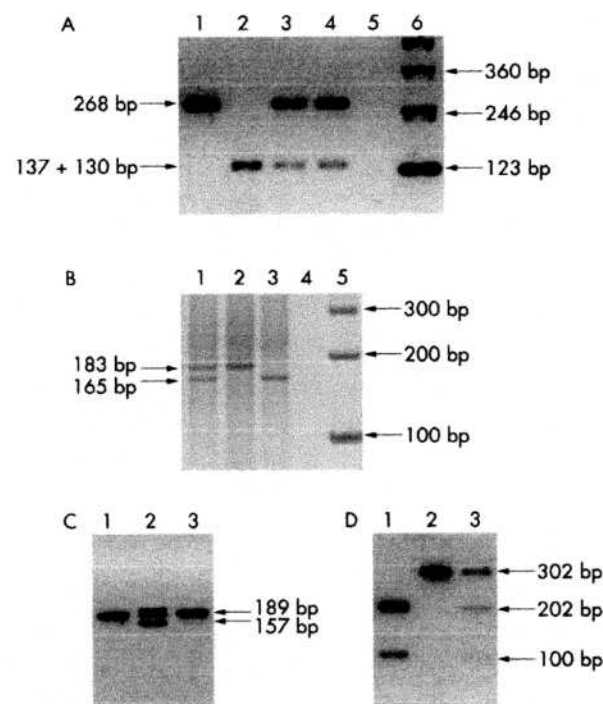


Figure 1 Representative samples from the RFLP genotype analysis. (A) Agarose electrophoresis for CCR5 59029 G-A. Samples: 1, wild type homozygote; 2, mutated homozygote; 3-4, heterozygotes; 5, negative control; 6, marker. (B) Acrylamide electrophoresis for CCR2-64I. Samples: 1, heterozygote; 2, wild type homozygote A; 3, mutated homozygote G; 4, negative control; 5, marker. (C) Agarose electrophoresis for CCR5 Δ 32. Samples: 1 and 3 wild type homozygote; 2, heterozygote. (D) Agarose electrophoresis for CXCL12-3A. Samples: 1, wild type homozygote; 2, mutated homozygote; 3, heterozygote.

Table 1 Allelic and genotypic frequencies in the female control and female breast cancer patient groups

Polymorphism	Normal (%)	Breast cancer (%)	Significance
CCR5Δ32			
Number of samples	210	264	
Wild/wild	196 (93.3)	238 (90.1)	p = 0.21 NO
Wild/mutated	14 (6.7)	26 (9.9)	df = 2
Mutated/mutated	0	0	
Allele frequency	0.0333	0.0492	p = 0.226 NO
Hardy-Weinberg	YES	YES	df = 1
CXCL12			
Number of samples	212	264	
Wild/wild	101 (47.6)	98 (37.1)	p = 0.067 NO
Wild/mutated	92 (43.3)	136 (51.5)	df = 2
Mutated/mutated	19 (8.96)	30 (11.3)	
Allele frequency	0.3066	0.3712	p = 0.036 YES
Hardy-Weinberg	YES	YES	df = 1 >95%
CCR2-64I			
Number of samples	211	264	
Wild/wild	154 (73)	221 (83.7)	p = 0.017 YES
Wild/mutated	50 (23.7)	38 (14.4)	df = 2 >95%
Mutated/mutated	7 (3.3)	5 (1.9)	
Allele frequency	0.1517	0.0909	p = 0.004 YES
Hardy-Weinberg	YES	YES	df = 1 >99%
CCR5 59029 G-A			
Number of samples	177	264	
Wild/wild	46 (25.99)	60 (22.7)	p = 0.236 NO
Wild/mutated	89 (50.28)	154 (58.4)	df = 2
Mutated/mutated	42 (23.73)	50 (18.9)	
Allele frequency	0.4887	0.4811	p = 0.823 NO
Hardy-Weinberg	YES	YES	df = 1

- for CCR559029 G-A, PCR conditions as above, with annealing temperature at 60°C. The reaction product was digested with 10 units BSp1286I /reaction (NEBS) at 37°C for five hours.¹³

All PCR samples with an undigested result were submitted to redigestion with 20 units/reaction of enzyme and overnight incubation.

The products of the digestion (CCR5 Δ 32, CCR264I, and CXCL12-3A) were visualised by agarose gel electrophoresis (2%) and ethidium bromide staining. The CCR5 59029 G-A digest was visualised in acrylamide gel electrophoresis and silver staining.

Statistical analysis

Genotype frequencies for each polymorphism were evaluated using the Hardy-Weinberg equilibrium test. Allele frequencies and the prevalence of genotypes were determined for the study and control groups and compared by the χ^2 test. Furthermore, for each polymorphism the allele presence (homozygous or heterozygous) or absence, and the homozygosity (for either allele) or heterozygosity, were statistically determined. Statistical significance was defined as $p < 0.05$. All analyses were performed using SPSSv10 (SPSS, Chicago, USA).

RESULTS

Table 1 summarises allelic and genotype frequencies for the breast cancer, table 2 for the bladder cancer, and table 3 for the non-melanoma skin cancer groups

CCR5 Δ 32 and CCR5-59029G

Genotype frequency distributions were in agreement with the Hardy-Weinberg equilibrium in all sample groups. We established no statistically significant difference in allelic frequency or genotype frequency between the cancer groups and the corresponding control groups.

Table 2 Allelic and genotypic frequencies in the male bladder cancer patient and male control groups

Polymorphism	Normal (%)	Bladder cancer (%)	Significance
CCR5A32			
Number of samples	148	68	
Wild/wild	127 (85.8)	58 (85.3)	p = 0.919 NO df = 2
Wild/mutated	21 (14.2)	10 (14.7)	
Mutated/mutated	0	0	
Allele frequency	0.0700	0.0735	p = 0.923 NO df = 1
Hardy-Weinberg	YES	YES	
CXCL12			
Number of samples	148	68	
Wild/wild	67 (45.3)	31 (45.5)	p = 0.983 NO df = 2
Wild/mutated	71 (48)	32 (47.05)	
Mutated/mutated	10 (9.7)	5 (7.35)	
Allele frequency	0.3074	0.3088	p = 0.976 NO df = 1
Hardy-Weinberg	YES	YES	
CCR2-64I			
Number of samples	148	68	
Wild/wild	115 (77.7)	51 (75)	p = 0.324 NO df = 2
Wild/mutated	33 (22.3)	16 (23.5)	
Mutated/mutated	0 (0)	1 (1.5)	
Allele frequency	0.1115	0.1324	p = 0.532 NO df = 1
Hardy-Weinberg	YES	YES	
CCR5 59029 G-A			
Number of samples	126	68	
Wild/wild	34 (27)	11 (16.1)	p = 0.130 NO df = 2
Wild/mutated	55 (43.7)	39 (57.4)	
Mutated/mutated	37 (29.3)	18 (26.5)	
Allele frequency	0.5119	0.5515	p = 0.456 NO df = 1
Hardy-Weinberg	YES	YES	

CXCL12-3A

Genotype frequency distributions were in agreement with the Hardy-Weinberg equilibrium in all sample groups. Comparison of each of the cancer groups with the corresponding control group showed significant differences in the breast cancer group in the allelic ($p = 0.036$; $df = 1$) and marginally non-significant in the genotypic ($p = 0.067$; $df = 2$) frequencies. Statistical evaluation after genotype grouping showed a statistically significant difference between the control groups in the breast cancer ($p = 0.02$; $df = 1$) and also in the non-melanoma skin cancer ($p = 0.032$; $df = 1$) groups when compared for the presence (CXCL12-3A/CXCL12-3A, CXCL12-3A/wild) or the absence (wild/wild) of the allele. No other genotype grouping gave statistically significant results.

CCR2-64I

Genotype frequency distributions were in agreement with the Hardy-Weinberg equilibrium in all sample groups. Comparison of each of the cancer groups with the corresponding control showed significant differences in the breast cancer group both in the allelic ($p = 0.004$; $df = 1$) and the genotypic ($p = 0.017$; $df = 2$) frequencies. Evaluation after genotype grouping showed a statistically significant difference in the breast cancer group when compared for the presence (CCR2-64I/CCR2-64I, CCR2-64I/wild) or the absence (wild/wild) of the allele ($p = 0.004$; $df = 1$). The

Table 3 Allelic and genotypic frequencies in the skin cancer patient and control groups

Polymorphism	Normal (%)	Skin cancer (%)	Significance
CCR5A32			
Number of samples	361	110	
Wild/wild	326 (90.3)	103 (93.6)	p = 0.283 NO df = 2
Wild/mutated	35 (9.6)	7 (6.4)	
Mutated/mutated	0	0	
Allele frequency	0.0484	0.0318	p = 0.294 NO df = 1
Hardy-Weinberg	YES	YES	
CXCL12			
Number of samples	363	110	
Wild/wild	169 (46.5)	64 (58.1)	p = 0.097 NO df = 2
Wild/mutated	164 (45.2)	38 (34.5)	
Mutated/mutated	30 (8.3)	8 (7.2)	
Allele frequency	0.3085	0.2454	p = 0.071 NO df = 1
Hardy-Weinberg	YES	YES	
CCR2-64I			
Number of samples	362	110	
Wild/wild	271 (74.9)	74 (67.3)	p = 0.234 NO df = 2
Wild/mutated	84 (23.2)	32 (29.1)	
Mutated/mutated	7 (1.9)	4 (3.6)	
Allele frequency	0.1353	0.1818	p = 0.087 NO df = 1
Hardy-Weinberg	YES	YES	
CCR5 59029 G-A			
Number of samples	306	110	
Wild/wild	80 (26.1)	29 (26.4)	p = 0.720 NO df = 2
Wild/mutated	145 (47.4)	56 (50.9)	
Mutated/mutated	81 (26.5)	25 (22.7)	
Allele frequency	0.5016	0.4818	p = 0.614 NO df = 1
Hardy-Weinberg	YES	YES	

grouping of homozygotes (CCR2-64I/CCR2-64I, wild/wild) and heterozygotes (CCR2-64I/wild) showed a statistically significant difference ($p = 0.009$; $df = 1$). No other genotype grouping gave statistically significant results.

DISCUSSION

In the present study, we investigated the potential involvement of polymorphisms in the genes CCR5, CCR2, and CXCL12 as markers for genetic events contributing to the appearance of breast, bladder, and non-melanoma skin cancer. We observed a significant association for CXCL12 and CCR2 polymorphisms exclusively in breast cancer. The lack of association in the skin cancer and bladder cancer groups should be evaluated in view of the smaller population sample that was available. Our results demonstrate a complete linkage disequilibrium between the CCR5 59029 G allele, CCR5A32, and CCR2-64I, in agreement with previously published findings.^{12,13}

CXCL12 is the main ligand for CXCR4. CXCL12-3'A is a single base variant in the 3' untranslated region of the CXCL12 gene, which initially was reported as a factor delaying AIDS progression to death especially in the late stages of HIV infection.¹⁶ Our data indicated a significant association in the allelic frequency of the CXCL12 polymorphism with breast cancer. Further genotype grouping revealed a significant preference for the presence of a mutated CXCL12 allele in the breast cancer group (OR = 1.54; 95% CI from 1.05 to 2.26).

CCR2 is the only 7-transmembrane G protein-coupled receptor of CCL2. CCL2 is abundantly produced in a variety of inflammatory diseases, such as atherosclerosis and rheumatoid arthritis. The CCL2 gene is also expressed during the early stages of melanoma, and it is produced in metastatic lesions. CCR2-64I is an amino acid substitution in the transmembrane domain of CCR2. CCR2-46I has been investigated extensively in AIDS related studies and is believed to affect the availability of CCR5 through the formation of heterodimers.¹⁷ In our study, significant differences were determined when the genotype and allelic frequencies in the breast cancer and control groups were compared. Genotype grouping revealed significant breast cancer protection for the mutated allele (OR = 0.53; 95% CI from 0.33 to 0.84) and the heterozygote genotype (OR = 0.54; 95% CI from 0.33 to 0.89).

The CCR5Δ32 allele encodes a truncated product that is not expressed on the cell surface¹⁸ but remains in the endoplasmic reticulum. CCR5Δ32 molecules are capable of forming heterocomplexes with normal CCR5 in heterozygous individuals, retaining normal CCR5 molecules in the endoplasmic reticulum and reducing cell surface expression of CCR5.¹⁹ In the present study, the CCR5Δ32 allele had no association with any of the cancer groups studied. This could be attributed to the fact that we did not find any mutated homozygotes which, according to previous studies, exert the major functional defect. CCR5 59029G/A is an A versus G single nucleotide variant at base pair 59029G in the promoter region of the CCR5 gene. The G allele exhibits a 50% lower expression of CCR5 in vitro, and confers slower AIDS progression than the A allele by 3.8 years (when comparing homozygous genotypes). Statistical analysis revealed no significant differences in the genotype and the allelic frequencies, concurring to an absence of involvement of CCR5, in agreement with the results on CCR5Δ32.

Previous reports on AIDS related non-Hodgkin's lymphoma (NHL) demonstrated that the CXCL12-3'A chemokine variant was associated with approximate doubling of the NHL risk in heterozygotes and an approximately fourfold increase in homozygotes, whereas the CCR2-64I allele had no effect.²⁰ The CCR5Δ32 allele in homozygotes was highly protective against NHL.²¹ The proposed mechanism of CCR5-Δ32 protection against lymphoma was thought to involve reduced cell surface expression of CCR5 during the B cell transformation in NHL. Although our results with regard to CXCL12 in breast cancer agree, the mechanisms of chemokine involvement in NHL could be totally different, since NHL cells of lymphocytic origin are involved and it is well established that chemokines can have a direct effect on lymphocyte trafficking and development.

Chemokines are primarily responsible for leucocyte infiltration in cancer.²² Briefly, we can summarise the possible function of lymphocytes associated with tumours as two models. On one hand, they are a potential source of growth factors for tumour cells and angiogenic factors for endothelial cells. Recent reports on human tissues noted an association between the extent of macrophage infiltration of breast cancers and their degree of vascularity, which suggests that leucocytes may contribute to tumour angiogenesis and, hence, tumour survival. On the other hand, leucocytes associated with tumours may be residual evidence of the host's ineffective attempt to destroy the tumour immunologically. It was thought that by enhancing this response, tumour eradication could be initiated. Several chemokines have been used in animal models to elicit immune responses specific to tumours and resulting in tumour rejection. These include CCL2,²³ CCL5,²⁴ CCL1,²⁵ CCL20,²⁶ CCL21,²⁷ CXCL10,²⁸ and XCL1.²⁹

The relevance of chemokines to malignancy extends beyond their roles in leucocyte recruitment. Murine models

show that chemokine secretion by tumour cells themselves may influence angiogenesis³⁰ and tumour growth.³¹ There are also reports that some chemokines may act directly as growth factors on tumour cells. Recently, research has focused on the role of chemokines in protumourigenic activities and the secretion of proinflammatory cytokines that may facilitate breast cancer metastasis formation³² and contribute to disease progression.³³

In summary, chemokines have been heavily implicated in breast cancer during the later stages of cancer progression and metastasis. Our data indicate a possible role for CXCL12 and CCR2 in the initial stages of breast cancer. The minor dysfunction of the chemokine system caused by the polymorphisms, although not essential for survival, could be critical for immune surveillance and eradication of the spontaneously appearing transformed cells. Further studies in human and animal models are necessary to verify the above observation and establish a possible mechanism.

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