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Original Paper

Allelic Imbalance at Chromosome Region 11q23 in Cervical Carcinomas

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The long arm of chromosome 11 has received much scrutiny as a high frequency of deletions of various sites has been observed in different tumour types, indicating the presence of putative tumour suppressor genes. In the present study, 81 primary cervical carcinomas were examined for allelic imbalance (AI) using nine microsatellite markers, mapping to the chromosomal region 11q23.1 where the *ATM* gene is located. AI at any locus in the region was found in 34 of 81 (42%) tumours. AI frequencies varied from 12 to 31% for the different markers used, with the highest frequency at marker *D11S1294*. Based on the findings of 17 cases with restricted areas of deletions, four chromosomal regions of possible importance in cervical carcinomas could be distinguished. The first region is located between the markers *D11S1325* and *D11S1819*, the second region between *D11S2179* and *D11S1294*, the third region between *D11S1778* and *D11S1818* and the fourth region between *D11S1818* and *D11S1347*. The second region may thus contain part of the *ATM* gene. No association between AI of any marker and histopathological or clinical parameters was seen. When comparing the AI findings of the different loci with TP53 protein overexpression, the only significant association found was with *D11S2179* located within the *ATM* gene. The results indicate that a tumour suppressor gene (or genes) on chromosome 11q.23.1 may be involved in carcinogenesis of the cervix and the involvement of the *ATM* gene remains a possibility. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: allelic imbalance, cervical cancer, ataxia telangiectasia, TP53, chromosome 11
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INTRODUCTION

Carcinoma of the uterine cervix is a common malignancy in adult women. Epidemiological studies suggest that infection with human papillomavirus (HPV) seems to be the initiating event for cervical carcinogenesis [1]. It is conceivable, however, that additional, genetic aberrations are required for tumour initiation and progression. Previous studies have shown that loss of heterozygosity (LOH) at specific chromosomal sites is frequently associated with the inactivation of tumour suppressor genes [2]. The 11q23 region has received much scrutiny, as a high frequency of deletions, indicating the site of a putative tumour suppressor gene (or genes), has been observed in tumours of the cervix [3, 4], ovary [5], breast [6], colon [7] and skin [8]. Hampton and colleagues demonstrated that 62% of cervical carcinomas had allelic deletions on chromosome 11q [3]. The highest frequencies of LOH were observed with the polymorphic marker at the *D11S144* locus (52%) and the *APOC3* gene (43%), both of which map to 11q23 [1–3]. LOH has also been reported in 40% of cervical cancers at *D11S29*, which is located at 11q23.3 [4]. An interesting candidate gene in the 11q23.1 region is the ataxia telangiectasia (*ATM*) gene [9], which in its mutated form is responsible for the autosomal recessive disorder ataxia telangiectasia (AT). AT is characterised by a number of clinical symptoms, including cerebellar degeneration and predisposition to cancer, the latter also being notable in AT heterozygotes [10]. Cells lacking the ATM protein show a reduced and delayed activation of the tumour suppressor gene *TP53* in response to DNA damage induced by

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radiation [11]. Wild-type TP53 protein transactivates genes involved in cell-cycle arrest and indirectly induces either DNA repair or apoptosis. Reduced and/or delayed activation of the TP53 protein may thus lead to genomic instability and cancer. Mutation of the *TP53* gene is the most frequent genetic alteration found in human tumours and the mutation often results in an increased stability of the protein. In cervical carcinomas mutation in the *TP53* gene is rare, occurring in only 2% of cases. Elevated *TP53* protein expression has, however, been detected in 55% of tumours [12]. The TP53 protein level may change owing to binding to the E6 protein of HPV or to other viral or cellular proteins [13]. Furthermore, the level of wild-type TP53 protein may change as a result of alterations in regulatory elements such as the *ATM* gene [11].

The aims of the present study were to define the smallest region of overlap of putative deletions of 11q23.1 and to determine whether the *ATM* gene is a target for allelic imbalance (AI) in cervical carcinomas. Furthermore, we wanted to relate the results to TP53 status, histopathological and clinical parameters and to evaluate whether these chromosomal changes provide prognostic information.

PATIENTS AND METHODS

Materials

Tumour tissue and peripheral blood samples were obtained from 81 patients with cervical carcinomas admitted to the Norwegian Radium Hospital between 1989 and 1991. All samples were kept frozen at -70°C until the analyses were performed. The median age at diagnosis was 52.4 years (range 22–84 years). The median observation time of the patients without relapse was 55.1 months (range 7.7–71.1 months). The histopathological characteristics and clinical diagnoses of the patients are shown in Table 1. Haematoxylin–eosin-stained sections were used to evaluate the approximate percentage of tumour tissue in the sample used for DNA extraction. Samples containing less than 20% tumour tissue were omitted from the analyses. DNA was extracted from minced tumour tissue and peripheral blood leucocytes after proteinase K digestion using phenol/chloroform extraction in an ABI DNA extractor using standard methods. DNA samples were stored at 4°C.

DNA analysis

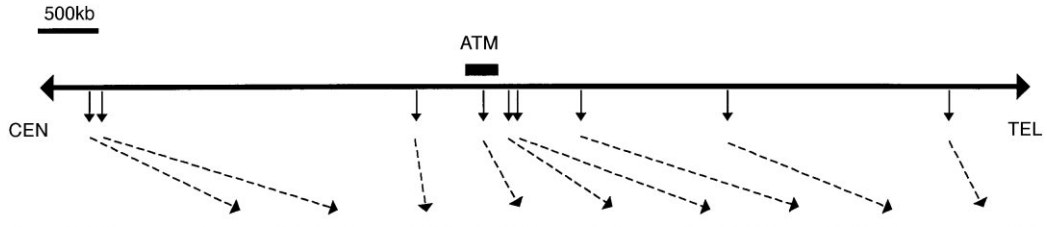
Nine loci mapping to chromosome region 11q23 containing dinucleotide repeat sequences were studied in matched blood and tumour pairs from 81 patients with cervical carcinomas. For seven of the samples only a limited amount of DNA was available and results were not obtained for all nine loci. AI frequencies are based on informative cases only. The microsatellite markers *D11S1325*, *D11S1347* and *D11S927* were selected from the Genome Database on the basis of localisation and polymorphic information content [6]. The microsatellite markers *D11S1816*, *D11S1819*, *D11S2179*, *D11S1778*, *D11S1294* and *D11S1818* have been identified and physically mapped to 11q23 [14, 15]. Microsatellite genotyping was performed by polymerase chain reaction (PCR) using 50 ng DNA as the template in a final volume of 10 µl with reaction concentrations of 200 µM each of dATP, dGTP and DTTP; 2.5 µM dCTP; 0.7 µCi [α-^{32}P]dCTP; 3 pmol of each primer (Research Genetics, U.S.A.) and 0.4 U DynaZyme (Version 2 F-500, Finnzyme, Espoo, Finland) in 1×DynaZyme buffer (1.5 mM $MgCl_2$). One PCR was performed combining the microsatellite markers *D11S1778* and *D11S1325*. The remaining loci were amplified separately. The reaction mixture was incubated in a PCR machine (PTC-100, MJ Research, San Fransisco, U.S.A.) for 27 cycles at 94°C (30 sec), 55°C (75 sec) and 72°C (15 sec). The reaction was initiated with a 5-min incubation at 94°C and ended with a 2-min incubation at 72°C. PCR products were electrophoresed under denaturing conditions and visualised through autoradiography as described previously [6].

All scorings were performed visually and independently by two of the authors. Either partial or total loss, or increased intensity of one allele in tumour DNA compared with the matched blood sample, was scored as AI. All cases with AI or microsatellite instability (MIN) were verified by analysing the samples twice. In samples with AI restricted to a limited region, flanking sequences with retained heterozygosity were

Table 1. *Histopathological and clinical diagnoses and allelic imbalance (AI) of chromosome* 11*q*23 *in cervical tumours*

Variable	Total no. of patients	Number of patients with AI/no. of heterozygote patients (%)								
		D11S1325	*D11S1816*	*D11S1819*	*D11S2179*	*D11S1778*	*D11S1294*	*D11S1818*	*D11S927*	*D11S1347*
AI for each marker		11/43 (26)	18/63 (29)	20/72 (28)	16/63 (25)	18/70 (26)	21/68 (31)	17/61 (28)	18/61 (30)	6/51 (12)
FIGO stage										
I	24	3/9 (33)	3/14 (21)	2/22 (9)	3/20 (15)	4/23 (17)	3/21 (14)	2/15 (13)	4/18 (22)	2/16 (13)
II	36	7/26 (27)	11/31 (35)	13/33 (39)	9/26 (35)	9/28 (32)	13/31 (42)	11/28 (39)	10/27 (37)	4/24 (17)
III	17	0/5 (0)	2/15 (13)	4/13 (31)	3/14 (21)	3/15 (20)	4/13 (31)	3/14 (21)	3/13 (23)	0/9 (0)
IV	4	1/3 (33)	2/3 (67)	1/4 (25)	1/3 (33)	2/4 (50)	1/3 (33)	1/4 (25)	1/3 (33)	0/2 (0)
Differentiation grade										
High	3	1/1 (100)	1/3 (33)	1/2 (50)	0/1 (0)	1/3 (33)	1/3 (33)	1/2 (50)	0/1 (0)	1/2 (50)
Moderate	53	6/29 (21)	13/43 (30)	16/48 (33)	12/41 (29)	13/45 (29)	14/44 (32)	10/41 (24)	12/39 (31)	4/32 (13)
Poor	20	4/13 (31)	4/17 (24)	3/22 (14)	4/21 (19)	4/22 (18)	6/21 (29)	6/18 (33)	6/21 (29)	1/17 (6)
Histological type										
Squamous cell carcinoma	64	10/34 (29)	15/51 (29)	17/58 (29)	13/48 (27)	16/55 (29)	17/54 (31)	14/59 (24)	15/50 (30)	5/40 (13)
Adenocarcinoma	8	1/4 (25)	1/6 (17)	1/7 (14)	1/6 (17)	2/7 (29)	2/6 (33)	1/4 (25)	1/4 (25)	1/6 (17)
Adenosquamous carcinoma	6	0/2 (0)	1/4 (25)	1/4 (25)	1/6 (17)	0/5 (0)	1/5 (20)	1/5 (20)	1/5 (20)	0/3 (0)
Other	3	0/3 (0)	1/2 (50)	1/3 (33)	1/3 (33)	0/3 (0)	1/3 (33)	1/2 (50)	1/2 (50)	0/2 (0)

FIGO, International Federation of Gynaecology and Obstetrics.



Sample	*D11S1325*	*D11S1816*	*D11S1819*	*D11S2179*	*D11S1778*	*D11S1294*	*D11S1818*	*D11S927*	*D11S1347*
42	AI	+	+	+	+	–	+	+	+
92	AI	AI	AI	AI	+	+	–	+	+
76	+	AI	+	+	+	–	+	+	+
182	+	AI	+	+	+	+	+	–	+
104	+	AI	AI	+	+	+	–	–	+
90	–	–	AI	AI	AI	+	+	–	AI
62	–	–	AI	AI	AI	AI	AI	AI	+
221	–	+	+	AI	AI	AI	+	AI	+
162	–	–	+	AI	AI	AI	–	AI	–
131	+	+	+	+	AI	+	+	–	+
181	–	–	–	+	AI	–	–	+	+
200	+	+	+	+	AI	AI	AI	+	+
137	–	+	+/MIN	–	AI	AI	AI	AI	AI
2	+	+	+	+	+	AI	AI	+	+
152	–	+	+	+	+	AI	+	+	–
158	–	+	+	+	+	+	+	AI	–
100	–	–	–	+	+	+	–	AI	AI

Figure 1. Cervical carcinomas with allelic imbalance (AI) of microsatellite markers located to chromosomal region 11q23.1. Only cases with AI of a restricted region are presented; all other cases had either no AI or AI of all informative cases. +, retained heterozygosity; –, homozygosity; MIN, microsatellite instability. ATM, ataxia telangiectasia gene; CEN, centromere; TEL, telomere. Darker shading represents regions with AI; lighter shading indicates regions of uncertainty.

confirmed by repeated analyses. AI in tumour tissue is a useful marker for putative allelic loss, although the method does not distinguish allelic deletions from mitotic recombination and allele amplification. Therefore, the term AI was used instead of LOH in this study. However, the frequency of AI may be an underestimate because markers were only scored as showing AI where the reduction in intensity was discernable by eye.

The results concerning AI of the *TP53* locus and immunohistochemical staining of the TP53 protein have been published [12]. AI of *TP53* was performed by Southern blot analysis using the probe pBHp53 and by PCR amplification of two polymorphisms (one in exon 4 and one in intron 6) within the *TP53* gene. Tissue sections were immunostained using the avidin–biotin–peroxidase (ABC) method with the monoclonal antibody PAb 1801 and the polyclonal antiserum NCL-CM1.

Statistical analysis

Differences in proportions were evaluated by the chi-squared or Fisher's exact test, as appropriate. Relapse-free survival was calculated from start of treatment to relapse or 31 Dec. 1996, using the method of Kaplan and Meier. The log rank test was used for univariate evaluation of relapse-free survival. Statistical significance was considered as $P < 0.05$. All P values are two-sided.

RESULTS

Allelic imbalance

Eighty-one primary cervical carcinomas were assessed for AI at nine microsatellite loci encompassing the 11q23.1 region. The results are summarised in Figure 1 and examples of tumours showing AI restricted to a limited region are shown in Figure 2. AI disclosed by at least one marker was detected in 34 (42%) of the 81 tumours examined. A total of 17 tumours demonstrated AI for all the informative markers, indicating loss of at least 10 Mb. The frequency of AI at the different loci varied from 12 to 31%. The lowest rate of AI was found at the most telomeric marker, *D11S1347* (12%). The highest frequencies of AI were found at the loci *D11S1294* (31%), *D11S927* (30%), *D11S1816* (29%) and *D11S1818* (28%). Among the 34 tumours exhibiting AI, 17 showed retention of heterozygosity of one or more markers indicating breakpoints of putative deleted areas (Figure 1). When considering tumour samples with AI of only restricted areas, AI was most often found to include *D11S1778* (8/17), *D11S1294* (7/14) and *D11S927* (6/13). The pattern of AI and retained heterozygosity indicated four critical regions, the first located between the markers *D11S1325* and *D11S1819*, the second between *D11S2179* and *D11S1294*, which could

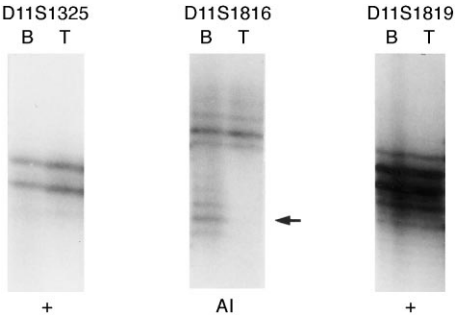


Figure 2. Example of allelic imbalance (AI) in cervical carcinomas: B, normal DNA from blood; T, tumour DNA. Areas representing putative DNA loss are marked with an arrow.

involve part of the *ATM* gene, the third between *D11S1778* and *D11S1818*, and the fourth region between *D11S1818* and *D11S1347*.

The locus *D11S2179*, which resides inside the *ATM* gene, demonstrated a total AI frequency of 25%. When considering tumours with restricted AI, the frequency was 5/16 (31%). None of the tumours had AI involving the *ATM* gene only.

The 81 cervical carcinomas have previously been analysed for AI of the *TP53* locus and for TP53 protein overexpression [12]. AI of *D11S2179* was not associated with AI of the *TP53* locus (only 2/16 cases with AI of *D11S2179* also showed AI of the *TP53* locus), but was associated with overexpression of *TP53* protein [13/16 cases (81%) with AI of *D11S2179* also showed overexpression of *TP53*, whilst 21 of 45 cases (47%) without AI of *D11S2179* showed TP53 protein overexpression ($P = 0.03$)]. There was no association of AI of the other markers on chromosome 11q with AI of the *TP53* locus or TP53 protein overexpression.

Each of three tumours exhibited novel alleles at one locus (*D11S1778*, *D11S1819* and *D11S1294*). The constitutional alleles were seen in addition to the new fragments in the microsatellite unstable tumours. In addition, one tumour showed MIN at four loci (*D11S1819*, *D11S2179*, *D11S1294* and *D11S1347*). The novel tumour alleles were seen at constitutional homozygous and heterozygous loci. Non-constitutional alleles were of shorter and intermediate size compared with the normal pattern.

Allelic imbalance and clinical parameters

There were no significant differences between cases with AI of chromosome 11q23.1 markers and cases without such changes regarding International Federation of Gynaecology and Obstetrics (FIGO) stage, histological type or grade of differentiation. Histopathological and clinical diagnoses and AI of chromosome 11q23 are presented in Table 1. There was a trend towards a decreased relapse-free survival time for patients with AI at *D11S2179* involving the *ATM* gene, although this was not statistically significant ($P = 0.09$). Patients who were heterozygous without AI of *D11S2179* had a survival rate of 53%, whilst patients with AI had a survival rate of 31%. There was no difference in survival for patients with or without AI at the other loci examined on 11q23.1.

DISCUSSION

In this study, AI for one or several markers was found in 34 (42%) of the tumours examined and the frequencies of AI for the different loci varied from 12 to 31%. Previously, AI of loci telomeric to the region examined in this work was found in 40% [4] and 62% [3] of cervical carcinomas. In one of these studies, allelic loss was estimated in 30% of tumours after excluding samples with a more general loss of a whole or hemichromosome 11 [4]. In the present study, 17 of the 81 tumours (21%) showed AI for all informative markers. However, only one of these tumours exhibited AI at the locus *D11S904* [16], which is situated on the short arm of chromosome 11. This implies that 20% of the tumour samples exhibited AI of a larger chromosome 11 segment, but the imbalance did not include the short arm, indicating that cervical tumours with loss of the whole chromosome 11 are not common.

As deduced from the pattern of AI and retained heterozygosity, the smallest region of overlap (SRO) of putative deletions did not define one region, but rather indicated four different locations of interest. A previous study [3] on LOH on 11q in cervical carcinomas showed the highest frequencies of LOH at markers *APOC3* (43%) and *D11S144* (52%), both telomeric to the loci investigated in the present study. The different areas of interest in these studies may therefore be separate AI targets, which may all play roles in the development or progression of cervical cancer.

The fact that the level of AI of *D11S2179* was not higher than that of the other markers does not rule out the possibility that aberrations of this locus are important in cervical carcinogenesis. However, there was no SRO that indicated that the *ATM* gene may be the sole target of inactivation. AI of *D11S2179* was not associated with AI of the *TP53* locus, but there was a positive association with overexpression of the TP53 protein ($P = 0.03$). The elevated level of TP53 protein was probably not caused by gene mutations, since the frequency of mutations demonstrated in this series was very low [12]. A relationship between loss of the *ATM* gene and increased TP53 protein level is in contrast to the previous observation of reduced and delayed activation of the *TP53* gene in cells lacking ATM protein. However, it has been shown that AT cells constitutively express a radiation-responsive protein and thereby behave as though they are in a continuous state of stress [17]. The fact that ionising radiation and oxidative stress are involved in the activation of the TP53 protein [18] may explain the findings of elevated levels of TP53 protein and AI of the *ATM* gene in the same tumours.

Only one tumour showed novel alleles at several loci, confirming previous results reporting a low frequency of microsatellite instability in cervical cancer [16, 19]. Even though this tumour may have originated in an individual with a defect in a mismatch repair gene, such aberrations are probably not common in cervical carcinomas.

There were no significant differences between cases with or without AI regarding histopathological or clinical parameters. However, there was a trend towards decreased survival of patients with AI of *D11S2179*. Previously, AI of another marker located at the 11q23 region was found not to correlate with the age of the patient at diagnosis or the histological tumour type [4].

In summary, based on the findings of 17 cases with restricted area of deletions, four chromosomal regions of possible importance in cervical carcinogenesis could be distinguished; one located between *D11S1325* and *D11S1819*, the second between *D11S2179* and *D11S1294*, the third between *D11S1778* and *D11S1818* and the fourth region between *D11S1818* and *D11S1347*. There was a significant correlation between AI of *D11S2179* and overexpression of TP53 protein [12].

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