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Analysis of Latvian melanoma families for *9p21* germline deletions by the multiplex ligation-dependent probe amplification approach

Dace Pjanova[1]*, Olita Heisele[1], Ludmila Engele[2], Juliette A. Randerson-Moor[3], Kairen Kukalizch[3], D. Timothy Bishop[3], Julia A. Newton Bishop[3]

[1]Biomedical Research and Study Centre, University of Latvia, Rātsupītes 1, Rīga LV-1067, Latvia
[2]Latvian Oncological Center, Hipokrāta 4, Rīga LV-1079, Latvia
[3]Genetic Epidemiology Division, Cancer Research UK Clinical Centre, St. James's University Hospital, Leeds, UK

*Corresponding author, E-mail: dace@biomed.lu.lv

Abstract

CDKN2A at chromosome band *9p21* is the most important melanoma susceptibility gene identified to date. Germline mutations of *CDKN2A* have been detected in melanoma families worldwide but the overall proportion of families with identified mutations remains moderate. Here we applied a novel method, called multiplex ligation-dependent probe amplification (MLPA), for detection of germline deletions at *9p21* in four melanoma-prone families from Latvia with no previously detected *CDKN2A* point mutations. No germline deletions were identified, excluding *9p21* deletions as a causal event in the patients analysed. However, we describe the application of MLPA and show the advantages of the method in gene dosage analysis.

Key words: *9p21*, *CDKN2A*, gene dosage, germline mutations, melanoma families.

Introduction

Many melanoma prone families show linkage to markers at chromosome region *9p21* (Newton Bishop et al. 1999) where the *CDKN2* locus is situated. The *CDKN2* locus is complex and consists of the *CDKN2A* and *CDKN2B* genes.

CDKN2A has been shown to be a melanoma susceptibility gene (Kamb et al. 1994). *CDKN2A* encodes the tumour suppressor protein p16 that acts through the retinoblastoma cell cycle control pathway (reviewed in Ortega et al. 2002). Germline mutations of *CDKN2A* have been identified in melanoma families around the world. However, the proportion of pedigrees in which *CDKN2A* is mutated remains moderate. Only approximately 25 % of families with two or more cases of melanoma have detectable mutations within the *CDKN2A* coding region (Goldstein, Tucker 1997). In the *CDKN2A* promoter region germline mutations occur rarely (Liu et al. 1999; Harland et al. 2000; Pollock et al. 2001). Recently, the lack of detectable mutations has been partly explained by the observation of noncoding mutations deep in the introns (Harland et al. 2001; Majore et al. 2004; Harland

et al. 2005a). However, there are still a significant proportion of *9p21*-linked families in which the susceptibility to melanoma remains unexplained.

The *CDKN2A* locus is complex and besides the above mentioned protein p16 gives rise to the second completely unrelated protein p14ARF through alternative splicing and translation of the products in different reading frames. The p16 and p14ARF mRNAs are transcribed from different first exons, 1α and 1β respectively, and utilise the same second and third exons. Like p16, p14ARF is also a tumour suppressor protein (Ortega et al. 2002). Mutations specifically altering *p14ARF* such as a *CDKN2A* exon 1β deletion (Randerson-Moor et al. 2001), a 16 bp insertion in exon1β (Rizos et al. 2001) and exon 1β splice site mutations (Hewit et al. 2002; Harland et al. 2005b) indicate a significant role of this transcript in melanoma predisposition.

No evidence has been found that *CDKN2B* (p15) is a melanoma susceptibility gene (Flores et al. 1997; Liu et al. 1997; Platz et al. 1997; Laud et al. 2006).

In other cancer family syndromes, germline deletions of susceptibility genes have been recognised as pathogenic mutations in a significant proportion of families. Germline deletions are common in *hMSH2* and *hMLH1* genes in hereditary non-polyposis colorectal cancer (Gille at al. 2002) and in the *BRCA1* gene in hereditary breast cancer (Petrij-Bosch et al. 1997). Germline deletions involving the *CDKN2A* locus have also been reported previously (Bauhuau et al. 1998; Mistry et al. 2005).

Here we applied a new technique, called multiplex ligation-dependent probe amplification (MLPA; Schouten et al. 2002), to the analysis of four patients from Latvian melanoma-prone families for heterozygous and homozygous germline deletions at *9p21*, and describe the value of the new technique for determination of the relative copy numbers of DNA sequences.

Materials and methods

Patients

The criterion for including patients in this study was the family history of melanoma. The information about families was obtained by personal interview of patients. Melanoma patients from four families (one patient from each family) with two or more cases of melanoma were analysed. Three patients were recruited through the Latvian Oncological Center and one patient was ascertained by referral from a clinician treating melanoma. All patients were previously analysed for germline mutations in the *CDKN2A* gene (exons 1β, 1α, 2, and 3) and did not have any detectable mutations (Pjanova, unpublished data). Informed consent approved by the Central Medical Ethical Committee of Latvia was obtained from all patients who underwent DNA analysis.

MLPA

Genomic DNA was extracted from peripheral blood lymphocytes by the standard phenol-chloroform extraction method.

MLPA was carried out according to the supplied protocol using the *9p21* MLPA probe kit from MRC-Holland, Amsterdam, the Netherlands. The technique and preparation of the probes used in this kit are described elsewhere (Schouten et al. 2002) and are outlined in Fig. 1. The *9p21* MLPA kit contains 12 probes for *CDKN2* locus sites, 11 probes for other *9p* gene sites, and 16 control probes specific for DNA sequences outside the *9p21* region.

The genes included in this analysis were *TEK*, *ELAV2*, *CDKN2B*, *CDKN2A*, *MTAP*, *KIAA*, *INFW1*, *IFNB1*, *MLLT3*, and *FLJ00026*.

Briefly, 25 ng μl^{-1} genomic DNA (in volume 2.5 µl) was denatured at 98 °C for 5 min, cooled to 25 °C, and 1.5 µl of the supplied SALSA probe mix and MLPA buffer were added. The mixture was re-heated to 95 °C and then the hybridisation was carried out at 60 °C

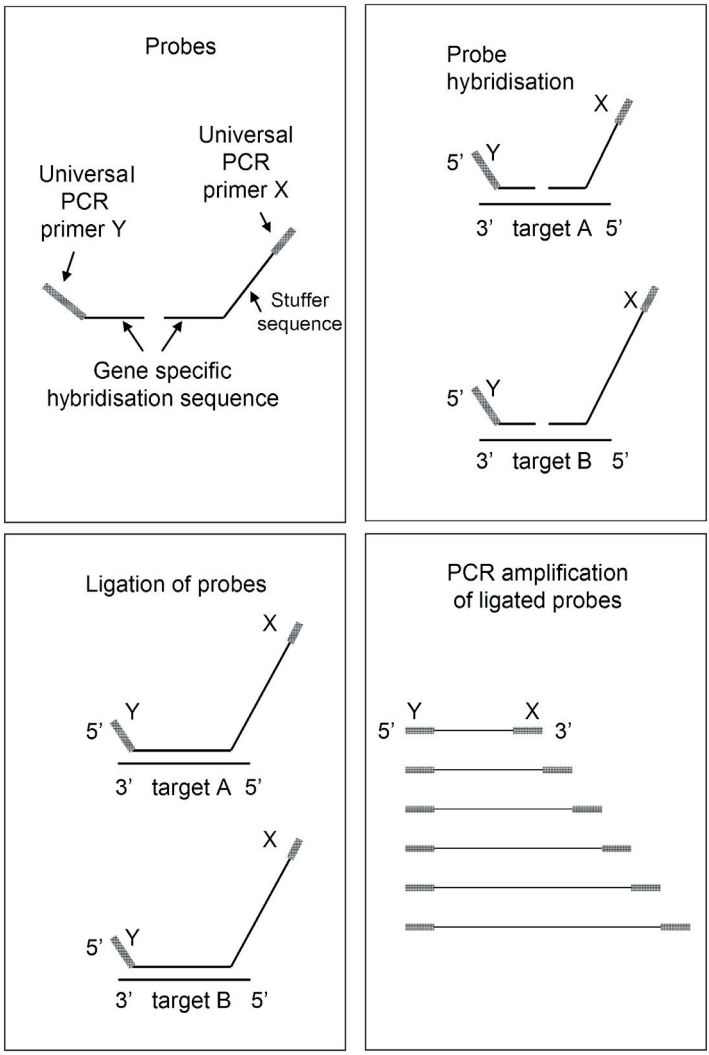

Fig. 1. Principle of multiplex ligation-dependent probe amplification (MLPA). For each specific target, a set of two probes is designed that hybridise immediately adjacent to each other. Both probes consist of a short target specific sequence and a universal forward or reverse PCR primer-binding site. In addition, one of the probes contains so-called stuffer sequence, which differs in length from probe to probe. After the hybridisation to the target sequence, the two parts of each probe are ligated by thermostable ligase. All probe ligation products are amplified simultaneously by PCR using a single primer pair. The multiple fragments can be distinguished based on their different length.

for 16 h. The hybridised probes were ligated with the ligase-65 mix (ligase-65 enzyme and ligase-65 buffers) at 54 °C for 10 to 15 min. The ligase-65 enzyme was inactivated by incubation at 98 °C for 5 min and the ligation products were than amplified by PCR according to the manufacturer's protocol using one primer labelled with 6-FAM. The amplification was performed on a GeneAmp 9700 Thermal Cycler (Applied Biosystems, Oxford, UK) with a hot-start PCR program beginning with the addition of the polymerase mix (SALSA primers, SALSA enzyme dilution buffer, SALSA polymerase) to the PCR reaction (ligation products premixed with SALSA PCR buffer) at 60 °C. PCR was carried out for 33 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, and incubation at 72 °C for 20 min.

PCR products (1 μl) were than mixed with 8.5 μl of de-ionised formamide (HiDi formamide, Applied Biosystems) and 0.5 μl fluorescent size standard (GeneScan-ROX 500, Applied Biosystems) and analysed on the ABI3100 Automated Capillary DNA sequencer with a 36 cm capillary array and ABI POP-4 polymer (Applied Biosystems). Analysis was automated using the ABI PRISM GeneScan and Genotyper software. Specific peaks corresponding to each product were identified according to their migration relative to the size standards and exported to a Microsoft Excel spreadsheet. To obtain gene dosage quotients (DQ), peak heights were taken as the quantitative measure of DNA content and peak fractions were calculated by dividing the peak area of a certain probe by the sum of peak areas of all 16 control probes in a certain sample. Subsequently, this relative peak area of each probe was compared to the average relative area of this probe in control samples. The means and standard deviations of the DQ provide quality control of the assay. Results from samples with mean standard deviation (SD) more than 0.2 were considered as false results and the analysis was repeated in accordance with the manufacturers recommendations.

Theoretically, gene dosage quotients close to 1.0 indicate two copies present (i.e. wild-type), 0.5, one copy absent (i.e. hemizygous), and 0.00, both copies absent (i.e. homozygous deletion). Quotients were scored according to observations made from large numbers of previous samples (data not shown): wild type if the quotient exceeded 0.75, hemizygous deletion if the quotient was between 0.43 and 0.68, and homozygous deletion if the quotient was between 0.00 and 0.19.

Results

We analysed DNA from four melanoma patients with a family history of melanoma for hemizygous and homozygous germline deletions at chromosome arm *9p*. Three patients (M66, M162, M199) were from two-case melanoma families and one patient (M247) was from a five-case melanoma family (data not shown). The MLPA assay was robust and reliable in most cases analysed, as seen by peak heights in control DNA samples across the multiplex (Fig. 2) and standard deviations in dosage quotients (Table 1). One DNA sample M162 did not provide acceptable data, producing high standard deviation variations in DQ estimates. However, after re-measuring of DNA concentration and increasing the DNA concentration in the experiment a high quality result was obtained (Table 2). No deletions were identified for any *9p21* gene probes in samples analysed (Table 2), suggesting that germline deletions at *9p21* are not responsible for clustering of melanoma in these families.

Discussion

Although some reports (Bauhuau et al. 1998; Randerson-Moor et al. 2001) have detected *CDKN2A* germline deletions in melanoma-prone families, such deletions explain susceptibility only in a small proportion of families. In the first comprehensive study carried out to date only 3 % of melanoma families (three families from 93) in which germline mutations have not been found have been shown to carry *CDKN2A* germline deletions. One family was reported to have a hemizygous deletion involving *CDKN2A* exons 1α, 2, and 3, and two families had hemizygous deletions of *CDKN2A* exon 1β (Mistry et al. 2005). In the present study we did not find any *9p21* (*CDKN2A*) germline deletions in Latvian melanoma-prone families, which might be explained by the rarity of such a deletions. It seems likely that there are as yet unidentified melanoma susceptibility genes, one of which may be at *1p22* (Gillanders et al. 2003). Taking together previous reports

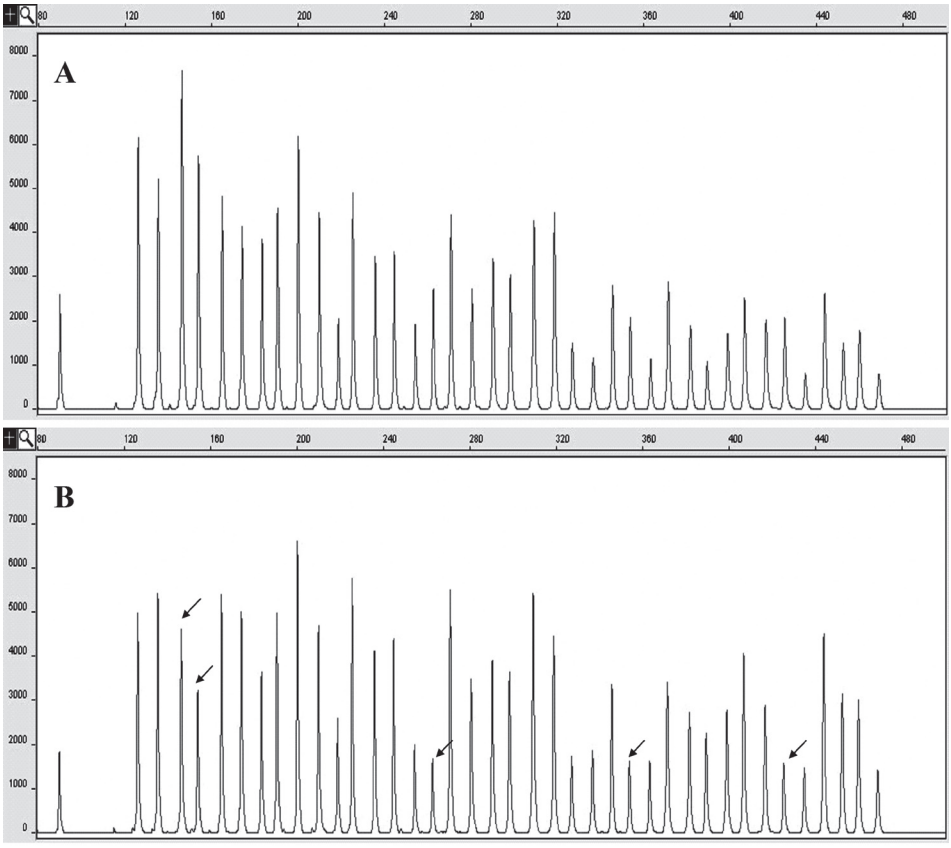

Fig. 2. GeneScan traces of MLPA products carried out on an automated capillary sequencer (ABI3100, Applied Biosystems). A, wild type control DNA. B, hemizygous deletion at *CDKN2A* exon 1β previously reported by Randerson-Moor et al. (2001) and used as a control in this study. Arrows indicate the fragments deleted in sample B..

Table 1. Gene dosage analysis by MLPA: typical dosage results showing a hemizygous deletion at *CDKN2A* 1β (bold). DNA from a melanoma pedigree previously reported by Randerson-Moor et al. (2001) and used as a hemizygous control in this study. [a], DQ (dosage quotient), the mean of DQs of each fragment against 16 control fragments. Values close to 1 indicate two copies of the fragment present and close to 0.5 loss of one copy of the fragment. [b], SD (standard deviation) used for quality control. Only results with SD ≤ 0.2 were taken into account. bp, base pairs

Fragment length (bp)	Probe	DQ[a]	SD[b]	Dosage
130	*C5q31*	0.75	0.10	Control
139	*C1p22*	0.94	0.13	Control
148	***CDKN2A* intron**	**0.53**	**0.07**	**Deletion**
157	***CDKN2A* 1β promoter**	**0.49**	**0.07**	**Deletion**
166	*MLLT3* exon 8	0.97	0.14	Normal
175	*C7p22*	0.94	0.13	Control
184	*MLLT3* exon 2	0.82	0.11	Normal
193	*C11p13*	0.96	0.13	Control
202	*CDKN2B* promoter	0.93	0.13	Normal
211	*CDKN2B* exon 1	0.95	0.13	Normal
220	*C14q24*	0.99	0.14	Control
229	*C5q35*	0.94	0.13	Control
238	*CDKN2A* exon 1	0.98	0.14	Normal
247	*C11q13*	1.06	0.15	Control
256	*CDKN2A* exon 2	0.83	0.12	Normal
265	***CDKN2A* 1β CpG island**	**0.46**	**0.06**	**Deletion**
274	*CDKN2A* exon 3	0.92	0.13	Normal
283	*C17p13*	0.99	0.14	Control
292	*MTAP* exon 7	0.97	0.14	Normal
301	*C8q24*	0.95	0.13	Control
310	*MTAP* exon 6	0.96	0.13	Normal
319	*FLJ00026*	0.82	0.12	Normal
328	*MTAP* exon 1	0.82	0.11	Normal
337	*C5q35*	1.00	0.14	Control
346	*C7q11*	0.92	0.13	Control
355	***CDKN2A* intron**	**0.68**	**0.10**	**Deletion**
364	*KIAA*	1.07	0.15	Normal
373	*C22q11*	0.98	0.14	Control
382	*IFNW1*	1.02	0.14	Normal
391	*CDKN2A* 1α promoter	1.29	0.18	Normal
400	*IFNB1*	1.12	0.16	Normal
409	*C2p14*	1.23	0.17	Control
418	*ELAVL2*	1.00	0.14	Normal
427	***CDKN2A* 1β exon 1**	**0.49**	**0.07**	**Deletion**
436	*TEK*	1.19	0.17	Normal
445	*C22q13*	1.09	0.15	Control
454	*CDKN2B* intron	1.44	0.20	Normal
463	*C10p14*	1.14	0.16	Control
472	*C8p23*	1.11	0.16	Control

Table 2. MPLA gene dosage quotients of patients from Latvian melanoma-prone families. [a], the probes are ordered from top to bottom across *9p21*, probes for *CDKN2* locus are highlighted in bold. DQ, dosage quotients. Values close to 1 indicate both copies of the fragment present. SD, standard deviation

	Probe[a]	Patients							
		M66		M162		M199		M247	
		DQ	SD	DQ	SD	DQ	SD	DQ	SD
Centromeric	*TEK*	1.10	0.10	0.85	0.10	1.49	0.26	1.36	0.16
	ELAVL2	1.13	0.10	0.91	0.11	1.27	0.22	1.07	0.13
	***CDKN2B* promoter**	**0.99**	**0.09**	**1.14**	**0.14**	**1.09**	**0.19**	**0.91**	**0.11**
	***CDKN2B* exon 1**	**0.96**	**0.09**	**1.17**	**0.14**	**1.01**	**0.18**	**0.87**	**0.10**
	***CDKN2B* intron**	**1.22**	**0.11**	**0.91**	**0.11**	**1.45**	**0.25**	**1.04**	**0.12**
	***CDKN2A* 1β CpG island**	**0.93**	**0.09**	**1.03**	**0.12**	**0.96**	**0.17**	**1.04**	**0.12**
	***CDKN2A* 1β promoter**	**1.03**	**0.10**	**1.15**	**0.14**	**1.00**	**0.17**	**0.96**	**0.11**
	***CDKN2A* exon 1β**	**1.22**	**0.11**	**0.84**	**0.10**	**1.03**	**0.18**	**1.22**	**0.14**
	***CDKN2A* intron**	**1.05**	**0.09**	**1.12**	**0.12**	**1.02**	**0.20**	**1.08**	**0.12**
	***CDKN2A* intron**	**1.02**	**0.10**	**1.15**	**0.13**	**0.97**	**0.18**	**0.95**	**0.13**
	***CDKN2A* 1α promoter**	**1.18**	**0.11**	**0.81**	**0.10**	**1.28**	**0.22**	**1.08**	**0.13**
	***CDKN2A* exon 1**	**1.09**	**0.10**	**1.08**	**0.13**	**1.06**	**0.18**	**0.98**	**0.11**
	***CDKN2A* exon 2**	**0.93**	**0.09**	**1.03**	**0.12**	**0.96**	**0.17**	**1.04**	**0.12**
	***CDKN2A* exon 3**	**0.92**	**0.08**	**0.96**	**0.11**	**0.92**	**0.16**	**0.92**	**0.11**
Telomeric	*MTAP* exon 7	0.97	0.09	1.10	0.13	1.02	0.18	1.04	0.12
	MTAP exon 6	1.07	0.10	0.99	0.12	0.99	0.17	0.87	0.10
	MTAP exon 1	0.94	0.09	0.93	0.11	0.84	0.15	1.11	0.13
	KIAA	0.96	0.09	0.99	0.12	1.16	0.20	1.00	0.12
	IFNW1	1.08	0.10	0.94	0.11	1.29	0.22	1.13	0.13
	IFNB1	1.09	0.10	0.91	0.11	1.21	0.21	1.05	0.12
	MLLT3 exon 2	1.01	0.09	1.13	0.13	0.89	0.16	0.90	0.11
	MLLT3 exon 8	1.09	0.10	1.14	0.14	1.08	0.19	0.81	0.09
	FLJ00026	0.98	0.09	1.07	0.13	0.93	0.16	1.06	0.12
	Mean SD		0.10		0.12		0.19		0.12

and results of this study, it is possible to exclude *CDKN2A* germline deletions as a major genetic determinant in melanoma susceptibility and to consider such a deletions as a rare event in melanoma-prone families.

The present study shows that MLPA is reliable, simple, and sensitive technique for relative quantification of nucleic acids, which is easy to perform and works as a multiplex assay. MLPA is a multi-step process, however the hands-on time is minimal, and detailed information is obtained. At present, different techniques are used for the detection of the copy number of genes including standard chromosome analysis, fluorescent *in situ* hybridisation (FISH), Southern blots, and loss of heterozygosity (LOH) assays. These methods are time consuming, difficult to use as a multiplex or require large amount of sample DNA (Southern blots). The PCR-based mutation detection methods are not able to

detect deletions and duplications when a normal allele is also present. The MLPA technique permits relative quantification of 40 different target sequences in a single reaction and only a thermal cycler and gene analyser or electrophoresis equipment are needed (Schouten et al. 2002). Ready-made commercial kits are available for a number of genes, as well as the tools to prepare custom kits. Moreover, the assay is suitable for high-throughput screening of DNA.

DNA concentration is important for high quality data. This would also be expected for any quantitative assay, as probe signal strengths depend on the relative amount of the target sequences present in the sample.

In summary, the present study shows that the MLPA assay is suitable for detecting deletions at *9p21*. It is sequence specific and sensitive. However, germline deletions at *9p21* are not responsible for melanoma susceptibility in Latvian melanoma-prone families, in which germline point mutations in *CDKN2A* have not been found. The susceptibility gene hunting must be continued.

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Delēciju analīze *9p21* lokusā ar MLPA metodi iedzimtās melanomas pacientiem Latvijā

Dace Pjanova[1]*, Olita Heisele[1], Ludmila Eņģele[2], Juliette A. Randerson-Moor[3], Kairen Kukalizch[3], D. Timothy Bishop[3], Julia A. Newton Bishop[3]

[1]Latvijas Universitātes Biomedicīnas Pētījumu un Studiju centrs, Rātsupītes 1, Rīga LV-1067, Latvija
[2]Latvijas Onkoloģijas centrs, Hipokrāta 4, Rīga LV-1079, Latvija
[3]Genetic Epidemiology Division, Cancer Research UK Clinical Centre, St. James's University Hospital, Leeds, UK
*Korespondējošais autors, E-pasts: dace@biomed.lu.lv

Kopsavilkums

Gēns *CDKN2A*, kas atrodas devītās hromosomas īsajā plecā *9p21*, ir galvenais šobrīd identificētais melanomas jutības gēns. *CDKN2A* mutācijas novēro melanomas pacientu ģimenēs visā pasaulē, tomēr ģimeņu skaits ar identificētām *CDKN2A* mutācijām joprojām ir neliels. Dotajā darbā meklēja iespējamās *9p21* reģiona delēcijas četrās Latvijas ģimenēs ar atkārtotiem melanomas gadījumiem, kurās mutācijas *CDKN2A* gēnā līdz šim nav atrastas. Analīzes veica, izmantojot jaunu MLPA (*multiplex ligation-dependent probe amplification*) metodi. Nevienā gadījumā nenovēroja delēcijas *9p21* reģionā. Tas norāda, ka analizētajos pacientos *9p21* delēcijas nav saistītas ar melanomas attīstību. Darbā arī aprakstīta MLPA metode un tās pielietošanas iespējas, kā arī parādītas un apspriestas metodes priekšrocības gēnu delēciju noteikšanā.