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SHORT REPORT

Multiplex single-tube screening for mutations in the Nijmegen Breakage Syndrome (*NBS1*) gene in Hodgkin's and non-Hodgkin's lymphoma patients of Slavic origin

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Patients with Nijmegen Breakage Syndrome (NBS) have a high risk to develop malignant diseases, most frequently B-cell lymphomas. It has been demonstrated that this chromosomal breakage syndrome results from mutations in the *NBS1* gene that cause either a loss of full-length protein expression or expression of a variant protein. A large proportion of the known NBS patients are of Slavic origin who carry a major founder mutation 657del5 in exon 6 of the *NBS1* gene. The prevalence of this mutation in Slav populations is reported to be high, possibly contributing to higher cancer risk in these populations. Therefore, if mutations in *NBS1* are associated with higher risk of developing lymphoid cancers it would be most likely to be observed in these populations. A multiplex assay for four of the most frequent *NBS1* mutations was designed and a series of 119 lymphoma patients from Slavic origin as well as 177 healthy controls were tested. One of the patients was a heterozygote carrier of the ACAAA deletion mutation in exon 6 (1/119). No mutation was observed in the control group, despite the reported high frequency (1/177). The power of this study was 30% to detect a relative risk of 2.0.
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Introduction

Nijmegen Breakage Syndrome (NBS) is an autosomal recessive chromosomal instability disorder similar to the ataxia telangiectasia (AT) resulting in microcephaly, growth retardation, and immunodeficiency. Malignancies of lymphoid origin at young age present with defects in the cell cycle check points and increased sensitivity to ionising radiation.[1–3] Moderately elevated cancer risk for heterozygote carriers of mutations in the *ATM* gene has been reported for leukemias, non-Hodgkin's lymphomas and breast cancer.[4]

The gene (*NBS1*) of a size of 56 kb is localised on 8q21.3, and codes for a gene product termed Nbs1, p95, or nibrin of 754 amino acids.[5] The protein is an essential part of a multifunctional complex involved in DNA double-strand break repair and response to radiation. Interactions of nibrin with *BRCA1* and *ATM/ATR* have been shown upon DNA damage.[6] Nibrin forms a complex with *Mre11,* and *Rad50 in vivo*,[7] which has suggested functions in both

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homologous recombination and nonhomologous end-joining and also a role in telomere maintenance. Nibrin is itself essential for some catalytic activities of this complex such as ATP-dependent partial unwinding of a DNA duplex[8] and the ATM-dependent activation (phosphorylation) of Chk2 upon gamma radiation.[9] The abrogation of this activation leads to entry into mitosis immediately after radiation suggesting that checkpoint defects may result from inability to activate Chk2. However, evidence for Nbs1-dependent phosphorylation of CHK2 has been obtained from some cell lines,[9] but not in others, where repair defects rather than a check point arrest have been proposed as a mechanism to increased radiosensitivity.[10] Nibrin has been shown to be essential in the radiation-induced phosphorylation of Mre11 (*human meiotic recombination 11*), by recruiting specific kinases.[11] Initially, the 657del5 allele was suggested to lead to a premature stop and a truncated protein giving a surprisingly mild phenotype, given that the mouse *NBS1* knockout leads to early embryonic lethality.[12] Recently, an alternative translation was shown to lead to a variant, possibly partly functional NBS1 protein.[13] Using screening techniques designed to detect carriers of the common mutations Varon *et al* found an unexpectedly high carrier frequency of the 657del5, which may contribute to cancer frequency in the studied Slav populations.[14] To verify that we designed a study for the simultaneous screening for the mutations in *NBS1* in the following positions: exon 6, 657 delACAAA, 698 delAACA, exon 7, 835delCAGA, 842 insT, and exon 10, 1142 delC.

Materials and methods

Subjects

Blood samples were obtained from 119 patients with Hodgkin's and non-Hodgkin's lymphoma. The recruited patients comprised of white Slavic Caucasians attended the 1st Department of Internal Medicine of the University Hospital in Prague. The following data on patients were retrieved from medical records: age, sex, clinical stage, presence of symptoms, nodal status, tumour size and grade, histopathological classification of tumour, performance status, international prognostic factor, chemotherapy, and result of chemotherapy. A control group was composed of 177 unrelated subjects of Caucasian origin. Controls were recruited mainly from staff of National Institute of Public Health, 3rd Medical Faculty, and inhabitants of houses for elderly citizens living in the same urban area as patients. Controls had no previous medical record of lymphoma or other cancer disease. The composition of control group was comparable to cases in terms of age and sex (83 females and 94 males, prevalence of subjects older than 50). Patients and controls were asked to read and sign an Informed Consent in agreement with requirements of the Ethical Commission of the National Institute of Public Health in Praha.

DNA extraction

Genomic DNA was isolated from peripheral lymphocytes by the phenol/chloroform extraction method followed by ethanol precipitation according to standard protocols.

Mutation screening

A single tube, multiplex PCR analysis of the major known mutations in *NBS1* was developed using the three primer pairs given in Table 1.

DNA (50–100 ng) were mixed with 30 pmol of each primer, 0.2 mM of each dNTP, 1.2 mM $MgCl_2$, PCR Buffer II from Perkin-Elmer ($MgCl_2$-free), and 0.75 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) in a final volume of 30 μl. After initial denaturation at 93°C for 30 s, amplification was accomplished using a protocol with 20 cycles including 30 s at 94°C, 30 s at 62–42°C with a decrement of 1° in each cycle, and 30 s at 72°C, followed by 10 cycles with annealing temperature 49°C in each cycle, with a final extension of 10 min at 72°C in a PE9600 cycler. The fragment lengths of the PCR products were 136 bp for exon 6, 140 bp for exon 7, and 160 bp for exon 10 of *NBS1* (Figure 1). Primers were labelled with FAM for exon 6, HEX for exon 7 and TET for exon 10. After completion of the multiplex PCR reaction with labelled primers, the mix was analysed by a single run of capillary electrophoresis on ABI PRISM™310. Running conditions at voltage 15 kV, 5 s injection time and a length of run of 24 min were set for optimal detection of the polymorphic alleles.

Table 1 Multiplex PCR for known mutations (column 2), PCR primer sequences and size of the product

Primer	*Position*	*Mutations*		*PCR (bp)*
NBSex6FAM	Exon 6	657 delACAAA 698 del AACA	5′-Fam CC ACC TCT TGA TGA ACC ATC TAT 3′ 5′ AAT TTA GCT TAT AAC ATA ATT ACC 3′	134
NBSex7HEX	Exon 7	835 delCAGA 842 insT	5′-Hex GG TTG ATA ACA GAA GAG AAT GAA GA3′ 5′GCA TAT CCA TTA TTG ACT GAA TCC3′	140
NBSex10TET	Exon 10	1142 delC	5′-Tet-TTT CTA CAG GGA TTT GAG TGA AAG 3′ 5′TCT CAT CTT AGC CAA AGT ATT TGA TACC 3′	160

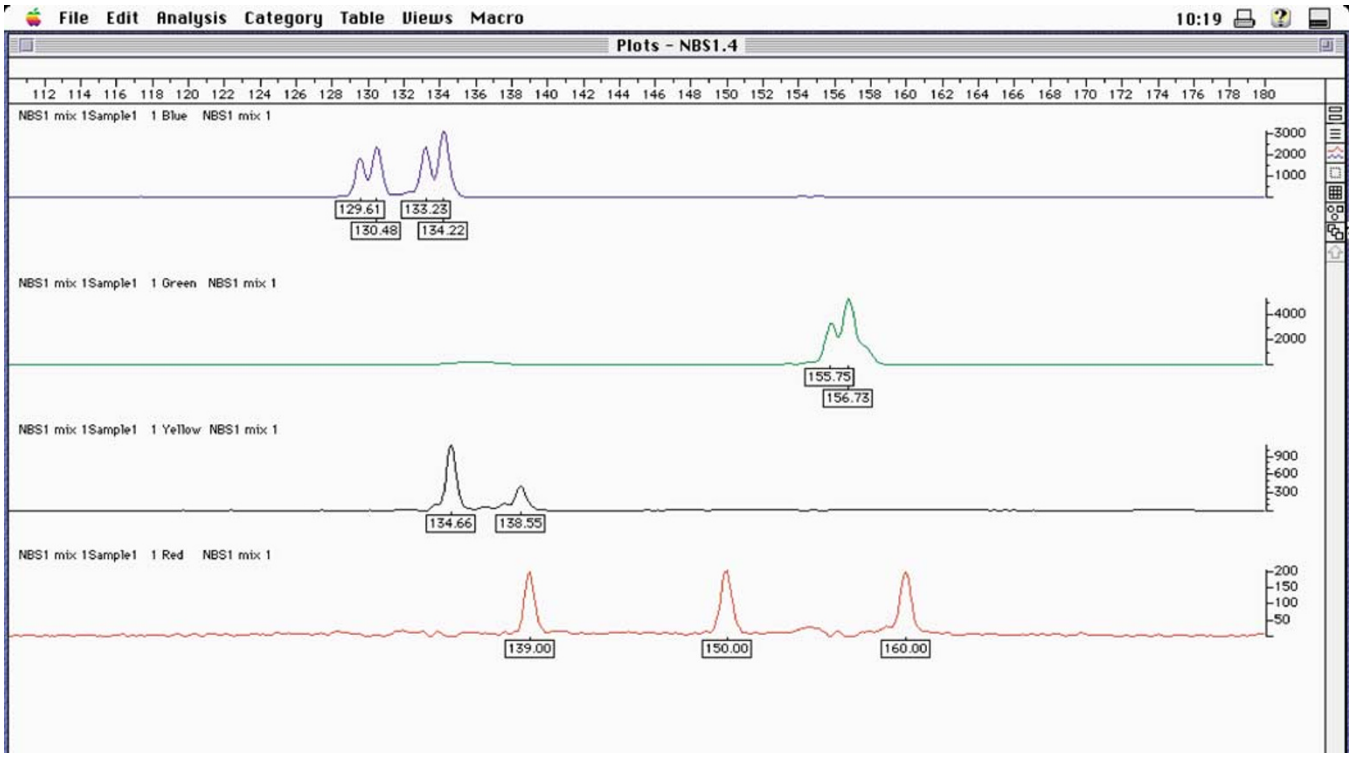

Figure 1 Multiplex PCR reaction was analysed by a single run of capillary electrophoresis on ABI PRISM™310. The fragment lengths of the PCR products, 134 bp for exon 6, 140 bp for exon 7, and 160 bp for exon 10 of *NBS1* for the homozygous wt are given relative to the internal standard. Primers were labelled with FAM for exon 6 (two blue peaks for a heterozygous deletion of 5 bp), HEX for exon 7 (two black peaks for a heterozygous deletion of 4 bp) and TET for exon 10 (two green peaks for a heterozygous deletion of 1 bp). Running conditions at voltage 15 kV, 5 s injection time and a length of run of 24 min were set for optimal detection of the polymorphic alleles.

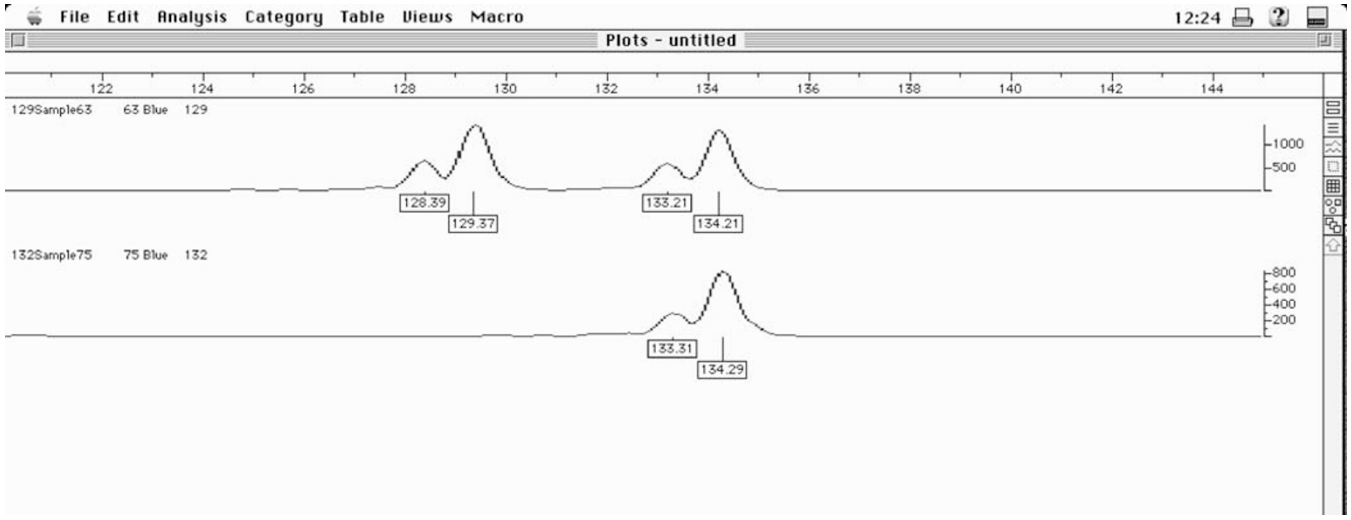

Figure 2 A non-Hodgkin's lymphoma patient presenting with heterozygote deletion of ACAAA exon 6.

Results

A single tube, multiplex PCR-based method for screening of the major known mutations in *NBS1* has been developed (Figure 1). Using this method we screened for mutated *NBS1* alleles in 119 lymphoma patients of Czech origin and 177 controls. In addition, the studied exons (exons 6, 7, and 10) have been amplified separately and sequenced to verify the results in 11 randomly chosen patients and 13

controls. One of the patients was a heterozygote carrier of the ACAAA deletion mutation in exon 6 (1/119) (Figure 2). No mutation was observed in the control group, despite the reported high frequency (1/177)[14] of the 657del5 mutation in Slavic populations. The present study does not therefore report an increased relative risk for lymphoma patients carrying the Slavic mutation although it was observed in one of the patients ($n=119$) and in none of the controls ($n=177$). The probability of finding no mutations, if the true probability in the population is P, can be calculated as follows (using the formula for the binomial distribution):

$$P(X=0)=\binom{n}{0}p^0(1-p)^{n-0}$$

In our example $n=119$ and $P=0.005$ (or 1/177) the probability of observing one mutation would be $0.995^{119}=0.55$. If the true probability was twice that observed in the control population ($P=2/177$) then it is $0.99^{119}=0.30$ ($P=0.01$), that is, our study had a 30% probability to detect an RR = 2.

Discussion

Both Hodgkin's and non-Hodgkin's lymphomas constitute a diverse group of malignancies with respect to histology, clinical presentation and disease progression. Genetic polymorphisms in xenobiotic metabolising enzymes of the cytochrome *P*450 and glutathione *S*-transferase superfamilies may contribute to disease risk.[15] The possible involvement of mutations in the *NBS1* gene in the pathogenesis of lymphoid diseases is suggested by the fact that this is the most common form of malignancy suffered by NBS patients.[1] Furthermore, mutations in the gene were found in some cases (14.9%) of childhood acute lymphoblastic leukemia[15] but not large deletions in the *NBS1* area on chromosome 8q.[17] Since at least one of the known variants, 657del5 in exon 6, was found in 1/177 healthy individuals of Slav origin,[14] we believed that it is feasible to expect a higher frequency of carriers of this mutation in lymphoma patients of the same population. Our results did not confirm this hypothesis in the studied patient groups. A larger set of both cases and controls is under investigation. However, the rapid and convenient screening technique that we introduce in this study could be used for larger population screening or for patient groups with increased radiosensitivity and abnormal response to radiation treatment.

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