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Cloning, expression, and mutation analysis of NOR_1, a novel human gene down-regulated in HNE_1 nasopharyngeal carcinoma cell line

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Abstract *Purpose*: To investigate cloning, expression, and mutation analysis of the putative candidate tumor suppressor gene related with nasopharyngeal carcinoma (NPC). *Methods*: We studied the expression profiles in the NPC cell line HNE_1 with the normal nasopharyngeal epithelial cell as control by using cDNA array representing 11,000 cDNA clusters. EST W95442 was found down-regulated in HNE_1. Subsequently, the corresponding gene sequence including this EST was established by cDNA cloning and the RACE (rapid amplification of cDNA end) procedure. The expression pattern of this gene was examined by using Northern blot analysis in various human tissues. Furthermore, we screened the mutations of the coding sequence of the gene using reverse transcription-polymerase chain reaction and single-strand conformation polymorphisms (RT-PCR-SSCP) as well as direct sequencing analysis. *Results*: A novel gene (GenBank accession No. AF462348) was cloned and named NOR_1 standing for oxidored-nitro domain-containing protein 1 (Human Gene Nomenclature Committee-approved symbol). Northern blot analysis revealed that the NOR_1 gene had two transcripts (1.2 kb, 1.6 kb), and expressed ubiquitously in human tissues. Moreover, a Glu58Gly mutation in the exon 1 of NOR_1 was detected in two of 25 NPC biopsies. *Conclusions*: We cloned a novel gene NOR_1, and the Glu58Gly polymorphism of NOR_1 may be involved in the development and/or progression of NPC suggesting that NOR_1 could be a candidate tumor repressor gene related with NPC.

Keywords Nasopharyngeal carcinoma · Gene cloning · Mutation analysis

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Introduction

Nasopharyngeal carcinoma (NPC) is an endemic cancer with a very high incidence in south-eastern China and North Africa (Hildesheim et al. 1993; Deng et al. 1998). There is a large body of evidence demonstrating that the intake of salted food and EBV infection are a major cause of the high incidence of NPC in these geographical regions (Yu at al. 1986; Poirier et al. 1989; Claudio et al. 2000). Several studies have shown that some samples of salted-fish contained relatively high levels of volatile nitrosamines, all of which induce tumors in the nasal cavities of experimental animals (Druckrey et al. 1967; Magee et al. 1976; Ward et al. 2000). Human are exposed through ingestion or inhalation of nitrosamines from the environment and the endogenous nitrosation of amino precursors in the body. In vivo mechanisms for the formation of nitrosamines may involve chemical and enzyme nitrosation, especially dependent on the presence of nitrate reductase and nitroreductase (Calmels et al. 1987; Zou et al. 1994; Mirvish et al. 1995).

As a high-throughput screening method, cDNA/EST array is an effective approach to finding new genes associated with diseases (Tomasinin et al. 2001). By means of the cDNA array, expression difference was detected by hybridization with cDNA probes from NPC cell line HNE_1 compared with normal nasopharyngeal epithelial cell in our earlier work. The down-regulated ESTW95442 was chosen because of further confirmation using Northern blot analysis. Subsequently, the

corresponding full-length cDNA was isolated and characterized (GenBank Accession number: AF462348). Here we describe the cloning and mutation analysis of this gene, designated NOR_1 standing for the oxidored-nitro domain-containing protein from human tissue (HUGO Gene Nomenclature Committee approved symbol).

Materials and methods

Specimens

Primary NPC biopsies were collected from Xiangya Hospital, Central South University, Changsha, Hunan, China. All NPC tumor biopsy specimens were classified histopathologically as primary nasopharyngeal tumors (NPC). None of the patients had received any chemo- or radiotherapy prior to biopsy. The primary tumor biopsies were snap-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. The NPC cell line HNE_1 was grown as monolayer in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Bioinformatics analysis

Database search was carried out using BLAST and Swiss-Prot. Domain search was performed with CDD (conserved domain database). All bioinformatic tools, including the open reading frame (ORF) identification mentioned in this paper, can be found as entries at ExPASY molecular biology WWW server of the Swiss institute of bioinformatics (www.expasy.ch).

5'RACE and clone sequencing

RACE was carried out with the Marathon-Ready brain cDNA RACE kit and Advantage PCR kit (Clontech, Palo Alto, Calif., USA) as described by the manufacturer. The gene-specific primer1 (GSP_1) (5'-CTCGGATGAATCCCTTGATGGTA-3') was 162–185 bp of cDNA clone 4816622 from the 5' end. The GSP_1 was used in combination with the adapter primer AP_1 to amplify the 5' end of the gene. The PCR amplification was performed for 32 cycles at 94 °C for 30 s and 72 °C for 4 min in a reaction volume of 50 μl according to the manufacturer's protocol. These PCR products were reamplified using a nested GSP_2 (5'-GCTGGAACTC CCCTGCAGAGAGA-3') and adapter primer AP_2. The reaction conditions were identical to first-round amplification. The final RACE product were subcloned to the PGEM-T system (Promega) and sequenced. All sequencing was performed on an ABI377 DNA sequencer (Perkin-Elmer, USA).

Expression analysis

For Northern blot analysis, a human multi-tissue Northern blot (Clontech) was hybridized with the 450 bp PCR product corresponding to the sequence of the coding region of NOR_1 according to the manufacturer's specifications. Probes were labeled with [^{32}-p] dATP using the Random primer kit (Boehringer, Mannheim). The filter was washed to a final stringency of 1×SSC, 0.1% SDS at 60 °C and exposed to film (Eastman Kodak) for 5 days at −80 °C.

Mutation analysis using RT-PCR-SSCP

cDNA synthesis was performed using 2 μg of total tissue RNA and 500 ng of oligo (dT) primer, using Expand Reverse transcriptase (Roche, Meylan France) according to manufacturer's recommendations. PCR amplification was performed with the aid of the following primers: S_1 (sense; 5'-ATGGTCAGGCCAAATC) and AS_1 (antisense; 5'-AAGTTCAAGAAGAGCAGC); S_2 (sense; 5'-AGCACCATGTCGGTGCG) and AS_2 (antisense; 5'-TAGAGCTCTTGAGGCTTG); S_3 (sense; 5'–AATAGAAAGTTTATGG) and AS_3 (antisense; 5'-ACACAGCAATACTTGAT); S_4 (sense; 5'-ACCTGATGACCATGGCT) and AS_4 (antisense; 5'-TCGGATGTGCGGTCTT); S_5 (sense; 5'-ATCCGGCAGACACTCCT) and AS_5 (antisense; 5'-TGGGTGCAGGGACATAG); S_6 (sense; 5'-ATGAATTCAAGCATGGTGG) and AS_6 (antisense; 5'-TCTTCTTTAGCAAGAGG). PCR amplification was carried out in the presence of 10% glycerol and consisted of 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a 10-min extension at 72 °C after the last cycle. Non-radioactive single strand confirmation polymorphism (SSCP) analysis was performed on all PCR products. Ten microliters of PCR product were mixed with an equal volume of denaturing solution (96% formamide, 20 mM EDTA, 0.05% bromophenol blue); the mixture was incubated at 98 °C for 10 min and rapidly quenched in ice. The samples were then applied to 8–10% polyacrylamide slab gels and electrophoresed at 150 V for 2–6 h. Gels were then sliver stained. PCR fragments displaying SSCP abnormal patterns were submitted to direct sequencing.

Results

Identification and cloning of the full-length cDNA of NOR_1

In a previous work, we used the cDNA array representing 11,000 cDNA to compare the expression profiles between HNE_1 cell line and normal nasopharyngeal epithelial cell. One EST (GenBank entry: w95442) was found with a high level of expression in primary culture of normal nasopharyngeal epithelial cells, whereas it was very low in NPC cell line HNE_1. The result of Northern blot hybridization of this EST (Fig. 1) was consistent with that of cDNA array analysis. This suggested that this EST might indicate a novel gene related with nasopharyngeal carcinoma, so we characterized it more fully.

To obtain the full-length cDNA, we obtained the cDNA clone 4816622 corresponding to the EST w95442. The sequencing of this clone includes an insert about 1.4 kb in length, a putative open reading frame (ORF) from nt 16 to 1107, and a poly(A) tail at the 3' end, but

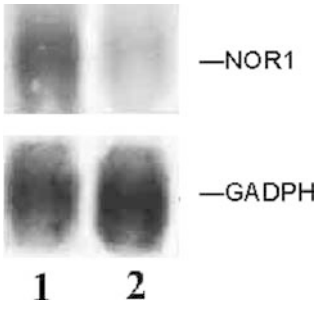

Fig. 1 Down-regulated expression of NOR_1 in NPC cell line HNE_1 compared with that in normal nasopharyngeal epithelial cell. *Lanes*: *1*, normal nasopharyngeal epithelial cell; *2*, NPC cell line HNE_1. GADPH served as controls for RNA quality

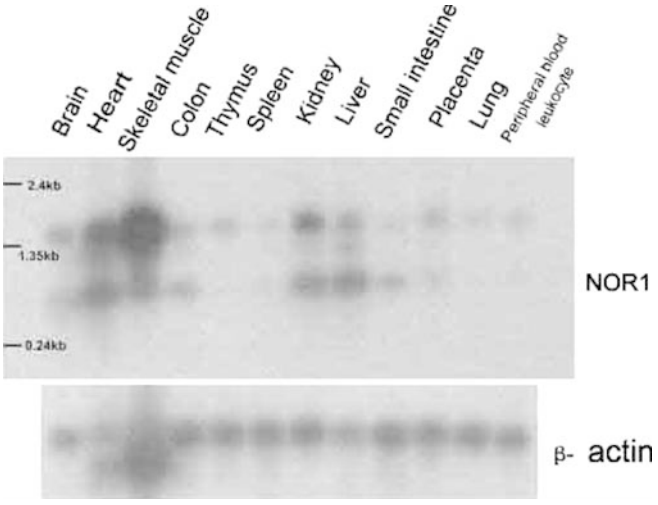

Fig. 2 Expression of NOR_1 mRNA in normal human tissues. A multitissue Northern blot (Clontech) was probed with NOR_1 cDNA. The same blot was rehybridized with a ß-actin probe, as a control for RNA loading and transfer

no in-frame stop codon at the 5' end. When this cDNA clone was used as a probe in the Northern blot, double transcripts (1.6 kb, 1.2 kb) were observed in all tissues studied and high expression levels were detected in skeletal muscle and heart (Fig. 2). Because the large transcript was about 200 bp longer than the cDNA clone insert, we used the Marathon-Ready brain cDNA RACE approach to find the 5' end as described above. Fortunately, the 350-bp fragment of RACE product was obtained and incorporated into the 5' sequence of the cDNA clone 4816622, and a 1601-bp gene sequence was generated (GenBank Accession No.AF462348).

Bioinformatics analysis of the sequence of NOR_1 cDNA

By searching NOR_1 gene in the Human Genome Database, we found that the NOR_1 is localized on chromosome 1p34.2 and has 10 exons and spans 33.4 kb of the genomic DNA. In addition, there was a putative open reading frame (ORF) from 33nt to 1298nt (Fig. 3) in NOR_1. The predicted protein has a theoretical molecular mass of 48 kD and a calculated isoelectric point of 5.78. The initiation codon (ATG in bold type) is surrounded by a translation initiation context (Kozak 1989). When NOR_1 was analyzed by search for functional domain using the bioinformatics tool (available at http:// www.ncbi.nlm.nih.gov/Structure/cdd/), the result revealed the presence of an oxidored-nitro domain in NOR_1. Interestingly, the deduced amino acid sequence of NOR_1 shares 39% identity with that of "classical" nitroreductase of *Salmonella typhimurium* (Fig. 4), so we proposed that NOR_1 is a novel nitroreductase derived from human tissue. In addition, the PROSITE database identified two possible cAMP and cGMP-dependent protein kinase phosphorylation sites, five Casein Kinase II phosphorylation sites, and four N-myristoylation sites in NOR_1.

a

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ataacttcgtatagcatacattatacgaagtt
                                   33atggatcaggccaaatcggccgagctcg
                                     M  D  Q  A  K  S  A  E  L  E
  61 aattcgtcgagagcggattgggacgctgcggcctggccttcaggccactggctaccgaac
       F  V  E  S  G  L  G  R  C  G  L  A  F  R  P  L  A  T  E  P
 121 cccggggctcttcaccagtccagctcgtttccagcaccatgtcggtgcggacgctaccgc
       R  G  S  S  P  V  Q  L  V  S  S  T  M  S  V  R  T  L  P  L
 181 tgctcttcttgaacttgggcggggagatgctttacatcctcgaccaacggctgcgggccc
       L  F  L  N  L  G  G  E  M  L  Y  I  L  D  Q  R  L  R  A  Q
 241  agaacatccggggagacaaggcccgcaaagttctgaatgacatcatctccaccatgttca
       N  I  R  G  D  K  A  R  K  V  L  N  D  I  I  S  T  M  F  N
 301 atagaaagtttatggaggaattattcaagcctcaagagctctactccaagaaggccctga
       R  K  F  M  E  E  L  F  K  P  Q  E  L  Y  S  K  K  A  L  R
 361 ggactgtctatgagcgcctggctcatgcctccattatgaaacttaaccaggccagcatgg
       T  V  Y  E  R  L  A  H  A  S  I  M  K  L  N  Q  A  S  M  D
 421 ataagctctatgacctgatgaccatggctttcaaatatcaagtattgctgtgtccccgac
       K  L  Y  D  L  M  T  M  A  F  K  Y  Q  V  L  L  C  P  R  P
 481 ccaaggatgtgctgctggtcactttcaatcacttggataccatcaagggattcatccgag
       K  D  V  L  L  V  T  F  N  H  L  D  T  I  K  G  F  I  R  D
 541 actccccaaccatcctgcagcaagtggacgagactttggcggcagctgacagaaatatatg
       S  P  T  I  L  Q  Q  V  D  E  T  L  R  Q  L  T  E  I  Y  G
 601 gtggtctctctgcaggggagttccagctgatccggcagacactcctcatcttcttccaag
       G  L  S  A  G  E  F  Q  L  I  R  Q  T  L  L  I  F  F  Q  D
 661 acctgcacatccgagtatccatgtttctaaaggacaaagttcagaataataacggtcgct
       L  H  I  R  V  S  M  F  L  K  D  K  V  Q  N  N  N  G  R  F
 721 ttgtgttgccggtgtccgggcctgttccttggggaactgaagttccaggactcatcagaa
       V  L  P  V  S  G  P  V  P  W  G  T  E  V  P  G  L  I  R  M
 781 tgttcaacaacaaaggtgaagaagtgaagaggatagaattcaagcatggtggaaactatg
       F  N  N  K  G  E  E  V  K  R  I  E  F  K  H  G  G  N  Y  V
 841 tccctgcacccaaagaaggttcttttgaactttatggagaccgagtcctgaaactgggaa
       P  A  P  K  E  G  S  F  E  L  Y  G  D  R  V  L  K  L  G  T
 901 ctaacatgtacagcgtgaatcagcctgtggaaactcatgtgtctggatcatcaaagaact
       N  M  Y  S  V  N  Q  P  V  E  T  H  V  S  G  S  S  K  N  L
 961 tagcctcatggacccaggaaagcattgctccaaaccctcttgctaaagaagagctgaatt
       A  S  W  T  Q  E  S  I  A  P  N  P  L  A  K  E  E  L  N  F
1021 tcttggccaggctgatgggagggatggagattaagaaacccagtggccctgagcccggat
       L  A  R  L  M  G  G  M  E  I  K  K  P  S  G  P  E  P  G  F
1081 tccggttgaatctctttaccaccgatgaagaagaggaacaagcagcgctaaccaggccag
       R  L  N  L  F  T  T  D  E  E  E  E  Q  A  A  L  T  R  P  E
1141 aagagttatcctatgaagttatcaacatacaagccacccaggaccagcaacggagcgagg
       E  L  S  Y  E  V  I  N  I  Q  A  T  Q  D  Q  Q  R  S  E  E
1201 agctggctcgaatcatgggggagtttgagatcacggagcagccaaggctgagcaccggca
       L  A  R  I  M  G  E  F  E  I  T  E  Q  P  R  L  S  T  G  K
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b

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1261 aaggggacgatttgctcgccatgatggatgagttatag1298
       G  D  D  L  L  A  M  M  D  E  L
ctgttctgacc ggcgtcctct gcccccaggg agaggctgct ggatggtgac ccctggggaa tgccccatgg
cccagaatga tgctgctagt tttctactga gtgaagccat tacgtctatt tcttatttat gttgtaagga
actgtgtgag tctcccttga ggagcactca ctcttgaagg cacacacata cacatatttt cagtgaaata
tattctgact tttaaacttg aaaaaaaaaa aaaaactctc cagcgctgga tccggccata agggcctgat
ccttcgaggg ggggcccggt a1601
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Fig. 3 Nucleotide sequence and the deduced amino acid sequence of the NOR_1 cDNA. The ATG start codon, TAG stop codon, and poly(A) tail are shown in bold. The tandem oxidored-nitro domains are underlined

Mutation analysis of the coding region of NOR_1 using RT-PCR-SSCP

We searched for mutations in the NOR_1 coding sequence using an RT-PCR approach. RNAs extracted from the 25 tumor biopsies were reverse-transcribed and cDNA used as a template for subsequent PCR amplification. Then, we used PCR-SSCP to detect mutations in the coding sequence of NOR_1. To this end, six overlapping PCR fragments were designed in order to

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Query: 169 RDSPTILQQVDETLRQLTEIYGGLSAG---EFQLIRQTLLIFFQDLHIRVSMFLKDKVQN 225
           R SP  LQ   + LRQ  E+   L AG   E QL   TLL   +D H  +S FL+ +  +
Sbjct: 196 RHSPLALQAAKQALRQSQEV--ALQAGLAQERQLF--TLLAATEDRHEGISAFLQKRTPD 251

Query: 226 NNGR 229
             GR
Sbjct: 252 FKGR 255
```

Fig. 4 Comparison of the amino acid sequence of NOR_1 with those of "classical" nitroreductase of *Salmonella typhimurium*. *Query* the amino acid sequence of NOR_1; *Sbjct* the amino acid sequence of "classical" nitroreductase

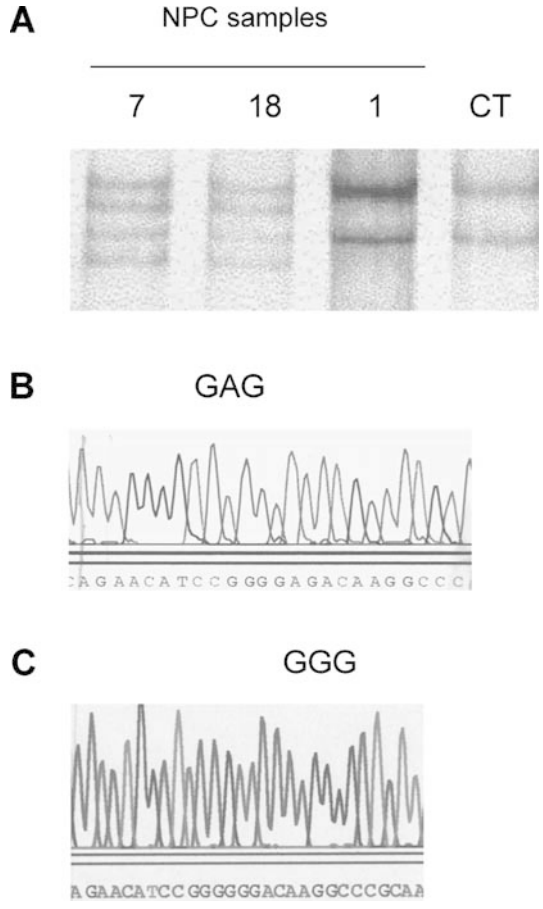

Fig. 5A–C Mutations in the NOR_1 gene detected by PCR-SSCP analysis in NPC samples. **A** PCR-SSCP analysis of exon1 of the NOR_1 gene from various NPC specimens and genomic placenta DNA as control (CT); **B** wild type sequence of NOR_1 from nt 241 to 264; **C** mutation sequence of NOR_1 from nt 241 to 264

amplify products with an average size of 200 bp. PCR-SSCP analysis of exon 1 showed the presence of two different migration patterns: the wild-type SSCP pattern exhibited by both the control placenta DNA and the sample NPC1, while the mutated pattern was found in the samples NPC7 and NPC18 (Fig. 5A). Interestingly, the two tumor samples NPC7 and NPC18 that exhibited the same patterns for exon 1 were shown to have the same mutations. The mutation corresponded to a GAG → GGG transition (Fig. 5B,C) at position 173 (exon 1) leading to the change of Glu into Gly at the amino acid residue 58.

Discussion

This paper reports the cloning and the mutation analysis of a novel gene NOR_1. NOR_1 gene encodes a putative protein of 421 amino acids with a theoretical molecular mass of 48 kD and a calculated isoelectric point of 5.78. The Northern-blot analysis showed the gene was expressed ubiquitously in human tissues. Because the predicted amino acid sequence has no significant homology to known proteins we were unable to speculate on the function of this protein. However, some bioinformatics tools employed in the PROSITE database identified an important oxidored-nitro domain in NOR_1 and we designated the novel gene as NOR_1 standing for oxidored-nitro contained protein from human tissue, which has been approved by HUGO Gene nomenclature Committee.

There is much evidence showing that nitrate reductases and nitroreductases are important enzymes during the formation of nitrosamines by their nitrosation activity (Ayanaba et al. 1973; Mills et al. 1976; Calmels et al. 1987). In addition, "classical" nitroreductase of *S. typhimurium* is a flavoprotein that catalyzes the reduction of nitroaromatics to metabolites that are toxic, mutagenic, or carcinogenic (Watanabe et al. 1998). The activity of "classical" nitroreductase to reduce a range of dinitrophenylcarboxamides has highlighted its potential usefulness in gene therapy for the tumor-selective activation of cytotoxic alkylating drugs (especially CB1954/NTR system) and has since been used in several prodrug approaches such as ADEPT (antibody-directed enzyme prodrug therapy), and GDEPT (gene-directed), (Parkinson et al. 2000; Plumb et al. 2001; Wilson et al. 2002). NOR_1 may be a novel member of nitroreductases which exhibit low substrate specificity but have the similar function of reducing nitro (Parkinson et al. 1998). Since NOR_1 may be a nitroreductase gene derived from human tissue, the functional study of NOR_1 may open the door to the study of relating environmental factors with genetic factors during the carcinogenesis of NPC.

Since only NOR_1 mRNA levels were measured in this study, the slow expression level observed could be due to a slow turnover of this messenger or its increased stability. Further, it is not known whether the NOR_1 protein levels are affected by the mutation or single nucleotide polymorphisms (SNP) of the NOR_1 gene. Investigations are currently underway to determine NOR_1 protein levels in nasopharyngeal carcinoma cell line HNE_1 and normal nasopharyngeal epithelial cell. Mutations (Glu58Gly) were found to occur in the coding region of NOR_1, which is most likely to influence the function of the gene. The hypothesis that mutation in the NOR_1 gene has an influence on the protein level or enzyme activity must certainly be extended further as has been suggested (Williams et al. 2001). Furthermore, chromosome 1p is the most frequent loss loci of genetic material by comparative genomic hybridization (CGH) in primary NPC biopsies (Guo et al. 1999; Yan et al.

2001; Li et al. 2001). Since the NOR_1 gene is located on 1p34.2 and has the genetic changes in the coding region, NOR_1 might be a good candidate tumor suppressor gene or related gene in nasopharyngeal tumorigenesis.

Large screening of the NOR_1 gene for mutations in patients from high-risk regions of NPC would help to clarify the relationship between the NOR_1 gene and NPC genesis. This would also help to clarify the role of dietary factors and/or EBV infections together with tumor suppressor gene mutations in NPC pathogenesis. The hypothesis that mutations in the NOR_1 gene are involved in NPC pathogenesis must be certainly extended further. A study examining a large number of NPC biopsies, especially in pedigrees of the NPC family, would help to better investigate the role of NOR_1 in nasopharyngeal tumorigenesis. Additionally, because identical mutations were found in different tumor biopsies, further investigation would be worthwhile on the possible presence of a mutational ''hot spot'' in NPC, which could be useful to develop a rapid diagnostic and/or prognostic tool for these patients.

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