Acta Biochim Biophys Sin (2008): 54–62 | © The Author 2009. Published by The Institute of Biochemistry and Cell Biology in association with Oxford University Press on behalf of SIBS, CAS. All Rights Reserved. DOI: 10.1093/abbs/gmn006.

Inactivation of *LARS2*, located at the commonly deleted region 3p21.3, by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma

Wen Zhou[1], Xiangling Feng[1], Hong Li[1], Lei Wang[1], Bin Zhu[1], Weidong Liu[1], Ming Zhao[1], Kaitai Yao[1,2], and Caiping Ren[1]*

[1]Cancer Research Institute, Xiang-Ya School of Medicine, Central South University, Changsha 410078, China
[2]Cancer Institute, Southern Medical University, Guangzhou 510515, China
*Corresponding author: Tel, 86-731-2355066; Fax, 86-731-4360094; E-mail, rencaiping@xysm.net

Allelic loss of chromosome 3p, including the 3p21.3 region, is found in 95–100% of primary nasopharyngeal carcinoma (NPC) biopsies, suggesting that this region should harbor some tumor suppressor genes (TSGs) closely related to NPC development. Several TSGs located at 3p21.3, such as *RASSF1A*, *LTF* and *BLU*, have been demonstrated to be involved in NPC development. *LARS2* (leucyl-tRNA synthetase 2, mitochondrial) is another gene located in the chromosome 3 common eliminated region-1 (C3CER1) at 3p21.3. In this study, we focussed on the epigenetic and genetic alterations of *LARS2* in NPC. The mRNA expression of *LARS2* was detected in 36 NPC and 8 chronic nasopharyngitis (NP) tissues by semi-quantitative reverse transcription-polymerase chain reaction (RT–PCR) and real-time RT–PCR. Subsequently, the mutation, allelic loss, and methylation status of *LARS2* were analysed by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), homozygous deletion (HD) analysis and methylation-specific polymerase chain reaction in primary NPC tissues. No expression or down-regulation of *LARS2* was observed in 78% of primary NPC tissues. No mutations, assessed by PCR-SSCP and DNA sequencing, were found in the promoter region and exon 1 of *LARS2* in NPC tissues, whereas HD was detected in 28% of NPC specimens at the *LARS2* locus. In addition, hypermethylation of *LARS2* was found in 64% of NPC samples but only in 12.5% of NP biopsies. Our data indicate that inactivation of *LARS2* by both genetic and epigenetic mechanisms may be a common and important event in the carcinogenesis of NPC.

Keywords nasopharyngeal carcinoma; *LARS2*; homozygous deletion (HD); mutation; methylation

Received: July 21, 2008 Accepted: August 20, 2008

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy with a high incidence of 25–30 per 100,000 in Southern China and southeast Asia [1–3]. Tumorigenesis of NPC is a multi-step process involving several factors, including Epstein–Barr virus infection and accumulation of epigenetic and genetic alterations [4].

Genetic studies using comparative genomic hybridization have shown that chromosomal abnormalities are involved in NPC, such as losses on chromosomes 3p, 11q, 13q, 14q, 16q, 16p, 1p, and 22q, as well as gains on chromosome 12p, 1q, 3q, 8q, 5p, and 7q [5–8]. Among these chromosomal abnormalities, deletion on chromosome 3p is extremely important because 3p deletion is detected in almost 100% of small-cell lung cancer, renal cell carcinoma, and 95–100% of primary NPC biopsies, and even 75% of pre-cancerous lesions showed loss of heterozygosity (LOH) on 3p [9], implicating that 3p deletion is an early and critical molecular event in the carcinogenesis of NPC and 3p should contain some important tumor suppressor genes (TSGs) closely related to NPC development.

To understand the role of 3p deletion in the development of NPC, we investigated the expression levels of several genes located at 3p21.3, the most frequently rearranged region on 3p. Previous studies in our lab demonstrated that several NPC-related TSGs such as *RASSF1A*, *GNAT1*, *LTF*, and *BLU* located at 3p21.3 were frequently inactivated by promoter hypermethylation and/or LOH in NPC [10–13]. *LARS2* (leucyl-tRNA

synthetase 2, mitochondrial), first reported by Kiss *et al* in 1999, is another gene located in the chromosome 3 common eliminated region-1 (C3CER1) at 3p21.3. It is identified by using the sequence of two overlapping PACs from C3CER1 and encodes the precursor of mitochondrial leucyl-tRNA synthetase which catalyzes the charging of $tRNA^{Leu(UUR)}$ with leucine, an essential step in protein synthesis. It performs essential roles in group I intron RNA splicing as well as protein synthesis within the mitochondria and is indirectly required for mitochondrial genome maintenance [14].

In this study, we examined the expression level, LOH, mutation, and methylation status of *LARS2* by reverse transcription-polymerase chain reaction (RT-PCR), homozygous deletion (HD) analysis, polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), and methylation-specific polymerase (MSP) chain reaction in primary NPC tissues in order to investigate the genetic and epigenetic alterations and the possible role of *LARS2* in NPC.

Materials and Methods

Tissue and blood samples

Thirty-six poorly differentiated NPC biopsies of primary tumors were obtained from NPC patients with consent before treatment at the Hunan Cancer Hospital (Changsha, China). In addition, eight chronic nasopharyngitis (NP) tissues were also obtained from patients without NPC at the Hunan Cancer Hospital. Among these 36 NPC biopsies, 25 biopsies as well as their matched peripheral blood samples were utilized for HD, mutation, and methylation analyses of *LARS2*. All the specimens were reviewed by an otorhinolaryngologic pathologist. Fresh NPC or NP tissues were snap-frozen in liquid nitrogen and stored until required.

Detection of the mRNA expression level of *LARS2* by semi-quantitative RT−PCR

Total RNA was extracted by TRIzol reagent (Gibco BRL, Grand Island, USA) and 2 μg of total RNA was subjected to cDNA synthesis using Superscript First-Strand Synthesis Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Primers for RT−PCR were designed to span at least two exons to avoid contamination by PCR products amplified from genomic DNA (gDNA). The primer sequences are listed in **Table 1**. PCR reactions were performed in a thermocycler under the following conditions: 95°C for 5 min, followed by 32 cycles of 94°C for 40 s, 58°C for 40 s, 72°C for 40 s, and then extension at 72°C for 10 min. At the same time, GAPDH was amplified as an endogenous control. The PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide (EB) staining.

The intensity of each band was measured by Image Master VDS (Pharmacia Biotech, Piscataway, USA) and analyzed by VDS software version 2.0 for band quantification. The expression levels of *LARS2* in NPC and NP tissues were investigated after they were normalized by transforming them into two groups of ratios of the band intensity of *LARS2* over that of *GAPDH* of the same sample. Each RT−PCR reaction was carried out in triplicate.

Detection of the mRNA expression level of *LARS2* by real-time RT−PCR

The cDNA generated was used for real-time RT−PCR amplification with SYBR Green I PCR Kit (TaKaRa, Shiga, Japan) as recommended by the manufacturer. The reaction was carried out in a real-time PCR instrument (MX3000P, Stratagene, La Jolla, USA). The primers used for real-time RT−PCR were same as those used for RT−PCR. *β-actin* was amplified as an endogenous control. PCR conditions were 95°C for 90 s, followed by 40 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 40 s, and a final extension at 72°C for 5 min. A series of diluted cDNA samples were used as templates to generate the standard curves, and melting curve analysis (100 cycles of 45−95°C for 10 s) was also performed to verify the presence of a single amplicon.

DNA extraction

gDNA from NPC biopsies as well as NP tissues was extracted using an improved method of extracting high-molecular-weight DNA with phenol/chloroform as described elsewhere [15], with a little modification. Briefly, tissues were ground in liquid nitrogen, lyzed in 500 μl of Tris/EDTA/SDS at 55°C for 30 min and incubated with proteinase K (2 mg/ml) at 37°C overnight, followed by phenol/chloroform extraction and stored at −20°C. gDNA from peripheral blood lymphocytes was extracted using Universal Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's instructions.

Allelic loss analysis

To examine the allelic loss in the *LARS2* locus, we selected two microsatellite markers flanking the *LARS2* gene. Primers for amplification of microsatellite markers RH25266 and SHGC-12886 are available through the

Table 1 Summary of primer sequences, annealing temperature and PCR product size

Primer	Sequence ($5' \rightarrow 3'$)	Annealing temperature (°C)	Product size (bp)
RT–PCR/real-time RT–PCR			
LARS2	TTACTGGATGCCTGTGGATT	58	295
	CCCGTTGTGTTTGGACTTAC		
GAPDH	CTTTGGTATCGTGGAAGGACTC	58	498
	CTCTTCCTCTTGTGCTCTTGCT		
β-actin	TAAGGAGAAGCTGTGCTACG	58	459
	GACTCGTCATACTCCTGCTT		
SSCP			
Promoter (−164 to −18 bp)	CAGAGCAGTTAAAGGGCG	60	145
	GACACAGACAGACGGGGG		
Exon 1	TAAAGGGCGTGCCAGAGG	60	317
	GAGCCGGAGGAAGAGGAGA		
Allelic loss analysis			
RH25266	GGGAAGCCATCAGAGACACT	58	216
	CTGAAGGCAAAGAGACCATT		
SHGC-12886	CCTGAGGAACTGCAGGACTC	58	338
	ATTCATGTGAGGAAGTGGTGC		
MSP (−184 to 81 bp)			
Methylated	AGATATTAGAGTAGTTAAAGGGCGT	58	227
	GAACACTACCGAACCACGAA		
Unmethylated	AGATATTAGAGTAGTTAAAGGGTGT	58	232
	TAAACAAACACTACCAAACCACAAA		

genome database on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) (**Table 1**). The microsatellite markers were amplified from 50 to 100 ng gDNA extracted from 25 NPC tissues and their matched blood samples which were used as controls. Reaction was initiated at 95°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a final elongation at 72°C for 5 min. After amplification, 6–8 μl of the reaction mixture was mixed with 8 μl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heat denatured, chilled on ice, then electrophoresed on a 6% polyacrylamide gel containing 8 M urea. The DNA bands were visualized by silver staining. HD was scored if one of the alleles showed at least a 50% reduction in intensity in tumor DNA compared to that in the matched blood DNA.

Moreover, we also used real-time RT–PCR analysis to more accurately calculate the HD frequency of *LARS2* in NPC. Samples and microsatellite markers were the same as those mentioned above. *β-actin* was amplified as an endogenous control. PCR conditions were 95°C for 90 s, followed by 40 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 40 s, and a final extension at 72°C for 5 min. The initial relative copy number DNA was given by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{tumor} - \Delta Ct_{normal}$ and each $\Delta Ct = \Delta Ct_{target} - \Delta Ct_{reference}$. Alleles were considered as homozygously deleted if the highest value of calculated range was below 0.5 and hemizygously deleted if this value was below 1.0 [16].

Detection of *LARS2* gene mutations using PCR-SSCP and DNA sequencing analysis

DNA samples from 25 primary NPC tissues as well as their matched peripheral blood samples were subjected to PCR-SSCP analysis for screening mutations in the promoter region and exon 1 of *LARS2* gene. We designed two pairs of primers located at −305 to −50 bp and exon 1 of *LARS2*, respectively. The primer sequences are listed in **Table 1**. PCR amplification was carried out in 20 μl reaction volume containing 50 ng of gDNA template, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM primer mixture (forward and reverse primers), and 1 U of *Taq* polymerase. For PCR reactions, after initial denaturation at 95°C for 5 min, 32 cycles, each consisting of denaturation at 94°C for 30 s,

annealing at 60°C for 30 s, and elongation at 72°C for 30 s, were performed, followed by a final elongation at 72°C for 10 min. Then the PCR products were denatured in loading dye at 99°C for 8 min and separated by electrophoresis on an 8% non-denaturing polyacrylamide gel. The results were visualized after the gel was stained with 0.2% $AgNO_3$. PCR products of primary NPC tissues showing distinct PCR-SSCP patterns from those of their matched peripheral blood samples were purified by the PCR product purification kit (TaKaRa) and then sequenced on the 377 ABI PRISM DNA sequencer (Shanghai Invitrogen Company, Shanghai, China).

Methylation analysis by MSP

gDNA from primary NPCs and NP tissues was treated with bisulfite, similar to our previous methods [17,18]. gDNA (10 μg) was denatured with 0.3 M NaOH, mixed with 333 μl of freshly prepared solution (10 mM hydroquinone and 3 M sodium bisulfite), covered with paraffin oil, and then deaminated in the dark for 4 h at 55°C. Bisulfite-treated DNA was purified with purification columns (TaKaRa), desulfonated with 0.3 M NaOH at room temperature for 10 min, neutralized with ammonium acetate, precipitated by ethanol, and resuspended in 20 μl of Tris–EDTA buffer. In this method, the sodium bisulfite reaction converts unmethylated cytosine in DNA to uracil while leaving the methylcytosine unchanged, so that methylated and unmethylated alleles can be distinguished by MSP. The MSP primers of *LARS2* were designed using the MethPrimer-Design software (http://www.urogene.org/methprimer). Detailed information concerning MSP primers for *LARS2* is shown in **Table 1**. Bisulfite-modified gDNA (~100 ng) was amplified by MSP in a total volume of 20 μl containing 1× PCR buffer, 0.25 mM dNTPs, 0.2 μM specific primer mixture (forward and reverse primers) and 0.5 U Hotstart (HS) *Taq* polymerase (TaKaRa). The MSP parameters were: 95°C for 5 min, followed by 32 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and then an extension at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% EB-stained agarose gel. The unbisulfited DNA was used as negative control and MSP primers were tested for not amplifying any unbisulfited DNA.

Statistical analysis

Statistical analysis was performed using the Wilcoxon rank sum test, χ^2 test, and Student's *t*-test, when appropriate. In all analyses, SPSS 10.0 statistical software (SPSS, Chicago, USA) was used and the statistical significance level was set at $P < 0.05$.

Results

Down-regulation of *LARS2* in NPC tissues detected by RT–PCR and real-time RT–PCR

RT–PCR was performed to analyze the expression of *LARS2* at transcription level. *LARS2* expression was examined in 36 primary NPC tissues as well as in 8 NP tissues. The results showed that all the eight NP tissues expressed stable *LARS2* mRNA level, whereas no *LARS2* transcript was amplified in 28% (10 of 36) of NPC tissues and down-regulation of *LARS2* was detected in 50% (18 of 36) of NPC tissues, indicating that aberrant expression (loss plus down-regulation) was detected in 78% (28 of 36) of NPC tissues. Compared with NP tissues, the mRNA expression level of *LARS2* was significantly down-regulated in NPC tissues ($P = 0.019$) (**Fig. 1** and **Tables 2** and **3**). To more accurately detect the expression level of *LARS2* gene, real-time RT–PCR was also performed in 36 primary NPC tissues and 8 NP tissues. According to the real-time RT–PCR results, *LARS2* was found to be down-regulated significantly in NPC tissues ($P = 0.02$) (**Table 3**), when the overall *LARS2* mRNA expression was compared between NPC and NP tissues. To determine whether the expression level of *LARS2* in primary NPC tissues was associated with the clinical features of the NPC patients, we compared the expression level of *LARS2* with the gender and lymph node metastasis (**Table 4**). The expression status

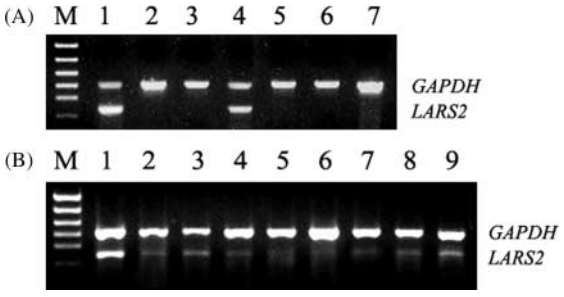

Fig. 1 Semi-quantitative RT–PCR analysis for detecting the mRNA expression level of *LARS2* in primary NPC tissues
Compared with NP tissues, primary NPC tissues expressed lower levels of *LARS2*. (A) Stable expression of *LARS2* was detected in NP tissue (lane 1) and primary NPC tissue (lane 4), but no *LARS2* transcript was detected in primary NPC tissues (lanes 2–3 and 5–7). (B) Stable expression of *LARS2* was detected in NP tissue (lane 1), but down-regulation of *LARS2* mRNA was detected in primary NPC tissues (lanes 2–9); M, DNA marker; *GAPDH* gene was used as an internal control.

Table 2 Genetic and epigenetic changes of *LARS2* in primary NPC tissues

Primary tumors ($n = 25$)	mRNA expression	Methylation	HD	Mutation
NPC-1	↓	M	ND	WT
NPC-2	↓	U	−	WT
NPC-3	L	M	+	WT
NPC-4	↓	U	ND	WT
NPC-5	↓	M	ND	WT
NPC-6	N	U	−	WT
NPC-7	↓	M	−	WT
NPC-8	↓	U	ND	WT
NPC-9	L	M	+	WT
NPC-10	↓	U	ND	WT
NPC-11	↓	M	−	ND
NPC-12	↓	M	ND	WT
NPC-13	↓	M	−	WT
NPC-14	N	U	−	WT
NPC-15	↓	M	+	WT
NPC-16	↓	M	ND	ND
NPC-17	N	U	−	WT
NPC-18	L	M	+	ND
NPC-19	↓	M	+	ND
NPC-20	↓	M	−	WT
NPC-21	↓	M	+	WT
NPC-22	N	U	−	WT
NPC-23	↓	M	−	WT
NPC-24	↓	M	+	ND
NPC-25	↓	U	−	WT

↓, decreased expression; L, lack expression; N, normal expression; M, methylated; U, unmethylated; ND, not detected; WT, wild-type.

of *LARS2* did not show any significant correlation with the gender ($P = 1.00$), whereas *LARS2* down-regulation was significantly correlated with lymph node metastasis ($P = 0.01$) (**Table 4**).

No mutations detected in the promoter region and exon 1 of *LARS2*

We analyzed mutations in the promoter region (−164 to −18 bp) and exon 1 of *LARS2* by PCR-SSCP and subsequent sequencing analysis in 25 primary NPC tissues and their matched blood samples. All the NPC samples tested showed the same mobility DNA bands as their matched blood samples. Further DNA sequencing revealed no mutations available in these two regions of *LARS2* gene (**Fig. 2**).

Allelic deletion of *LARS2* gene in NPC

Using two microsatellite markers, allelic deletion of *LARS2* was examined in 25 primary NPC biopsies and their matched blood samples. The results showed that HD frequency for RH25266 and SHGC-12886 was 16% (4 of 25) and 12% (3 of 25), respectively, resulting in a total HD frequency of 28% in NPC, which was further confirmed by real-time RT−PCR (**Fig. 3**). These findings demonstrated that allelic loss may be one of the mechanisms involving in the inactivation of *LARS2* in NPC.

Hypermethylation of *LARS2* gene in NPC

We analyzed the methylation status of 25 CpG islands in a 227-bp promoter region of *LARS2* in 25 primary NPC samples and 8 NP tissues by MSP [**Fig. 4(A)**]. Hypermethylation of *LARS2* promoter was only detected in 12.5% (1 of 8) of NP tissues; however, it was found in 64% (16 of 25) of primary NPC tissues [**Table 2** and **Fig. 4(B)**]. Statistical analysis indicated that there was a significant difference in methylation frequency of *LARS2* between NP and primary NPC tissues (χ^2 test, $P = 0.017$) (**Table 5**). Meanwhile, we also found that hypermethylation of *LARS2* showed a significant correlation with lymph node metastasis (Fisher's exact test, $P = 0.010$) (**Table 5**).

Table 3 Statistical analysis of RT−PCR and real-time RT−PCR results of *LARS2*

Group	Case no.	Mean age (years)	Gender		RT-PCR		Real-time RT-PCR	
			Female	Male	Mean ± SD[†]	*P*-value	$Z^{\ddagger}$	*P*-value
NP	8	48.2	3	5	0.381 ± 0.181	0.019	−2.331	0.020
NPC	36	49.5	9	27	0.821 ± 0.031			

Statistical results showed that mRNA expression of *LARS2* in NPC tissues was significantly lower than that in NP tissues. [†]Median of relative amount (intensity of *LARS2* over that of *GAPDH* of the same sample). [‡]Cycle threshold (Ct) in real-time RT−PCR. mean ± SD, *t*-test; *Z*, Wilcoxon rank sum test, $P < 0.05$ was regarded as statistically significant.

Table 4 Relationship between *LARS2* expression and clinical features of NPC patients

Group	Case no. ($n = 36$)	*LARS2*		*P*-value
		N	↓/L	
Gender				
Female	9	2	7	1.00
Male	27	6	21	
Lymph node metastasis				
Yes	28	3	25	0.01
No	8	5	3	

↓, decreased expression; L, lack expression; N, normal expression. Statistical analyses indicated that there was no significant correlation between *LARS2* expression and the gender of NPC patients, but *LARS2* down-regulation showed a significant correlation with lymph node metastasis (χ^2 test). $P < 0.05$ was regarded as statistically significant.

Discussion

Numerous studies have indicated the presence of TSGs on the short arm of human chromosome 3 involved in the development of many cancers, e.g. lung cancer, breast cancer, head and neck cancer, and ovarian cancer [19]. LUCA (also referred to 3p21.3C) and AP20 (also referred to 3p21.3T), two most frequently rearranged regions on 3p, were of high-frequency LOH or HD in multiple epithelial malignancies [16,20]. Besides, another region between D3S32 and D3S2354, which was located distal to LUCA, was also reported to be frequently deleted in lung and other cancers [21,22]. Although the first data suggesting that 3p deletions were involved in nasopharyngeal carcinogenesis had been published more than 10 years ago, only recently has significant progress been achieved in identifying the

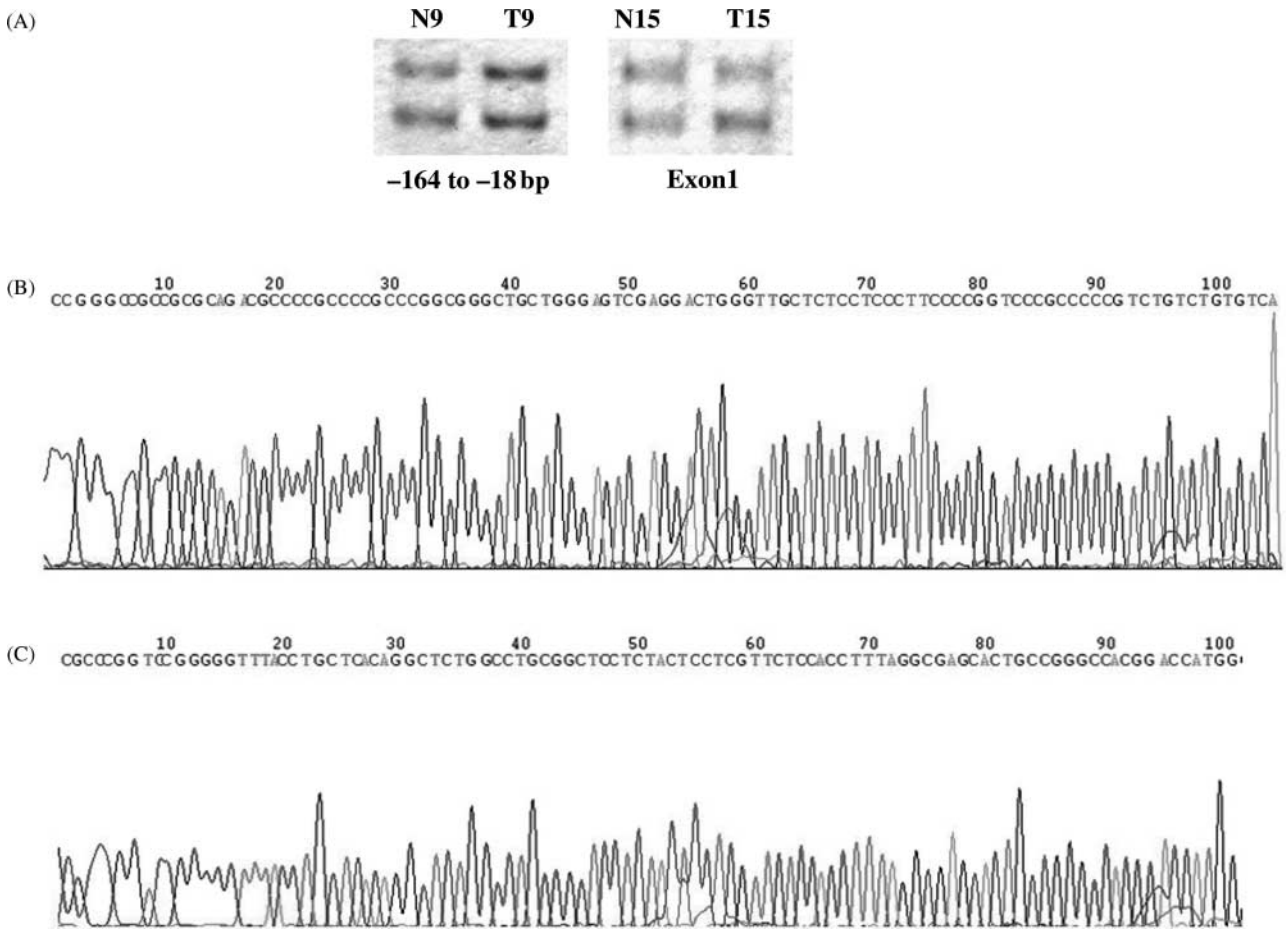

Fig. 2 Detection of mutations in promoter region, exon 1 of *LARS2* by PCR-SSCP and sequencing No mutations were detected in the promoter region and exon 1 of *LARS2*. (A) PCR-SSCP results. N, matched control peripheral blood samples; T, primary NPC tissues. (B) DNA sequencing results for promoter region −164 to −18 bp. (C) DNA sequencing results for exon 1.

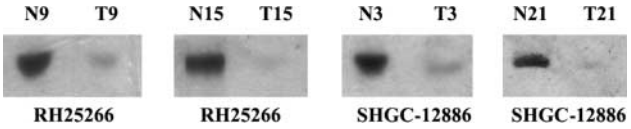

Fig. 3 Representative results illustrating HD of *LARS2* using two microsatellite sites (RH25266 and SHGC-12886) HD of *LARS2* was detected in the primary NPC tissues, compared with the matched control peripheral blood samples. N, matched control peripheral blood samples; T, primary NPC tissues.

candidate TSGs and demonstrating their functional roles in NPC development. At present, some genes located on 3p have been considered as promising candidate NPC-associated TSGs, such as *RASSF1A*, *BLU*, *LTF*, and *DLEC1* [23]. It has been demonstrated that genetic and epigenetic abnormalities of these promising candidate NPC-associated TSGs residing in chromosome

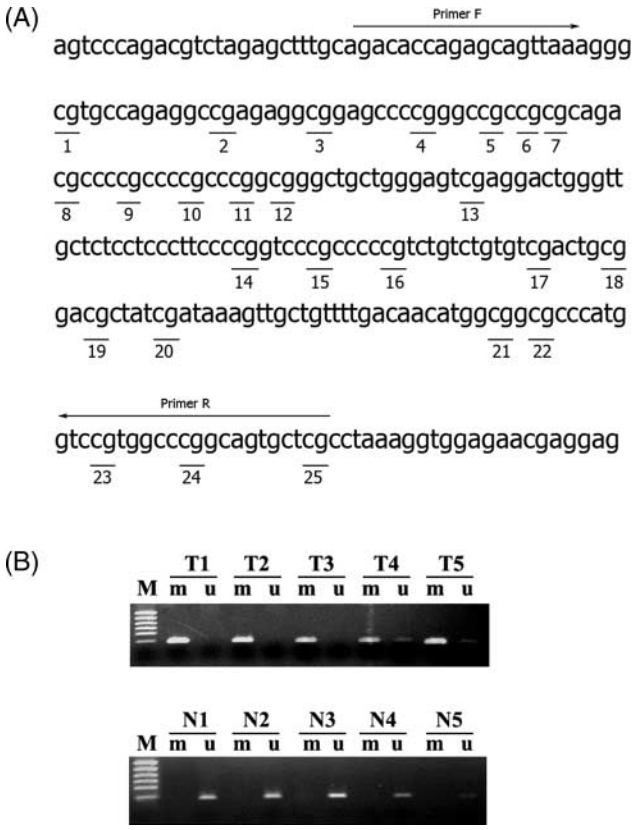

Fig. 4 The sequence of *LARS2* promoter region and the methylation status of *LARS2* CpG islands in NPC tissues analyzed by MSP (A) Positions of the primers used for MSP analysis along with CpG sites of *LARS2* gene. The positions of CpG sites are numbered 1–25. Horizontal arrows indicate the positions of MSP primers. (B) MSP analysis of *LARS2* in primary NPC and NP tissues. m, MSP products using methylation-specific primers; u, MSP products using unmethylation-specific primers; N, NP tissues; T, primary NPC tissues.

Table 5 Relationship between *LARS2* promoter methylation and clinical features of NPC patients

Group	Methylation of *LARS2*		*P*-value
	+	−	
Histological type			
NP tissues	1	7	0.017
NPC tissues	16	9	
Lymph node metastasis			
Yes	14	3	0.010
No	2	6	

$P < 0.05$ was regarded as statistically significant.

region 3p may be important for the development of NPC; however, it is still obscure how many of them exist and which of the numerous candidate TSGs are the key players in NPC pathogenesis.

LARS2 gene is located at the 1-Mb long common eliminated region 1 (CER1) between D3S32 and D3S3582 at 3p21.3 and spans 160 kb consisting of 22 exons encoding a 903-amino acid protein (Locuslink ID 23395). It is often found to be up-regulated in the brains of patients with bipolar disorder and schizophrenia and it may represent a novel type 2 diabetes susceptibility gene [24,25]. However, there is no literature reporting its role in tumorigenesis. In this study, we detected its expression level and genetic and epigenetic alterations in NPC tissues. To our knowledge, this is the first report showing association between down-regulation of an aminoacyl tRNA synthetase gene and NPC tumorigenesis.

According to the semi-quantitative RT–PCR and real-time RT–PCR results, aberrant expression (loss plus down-regulation) of *LARS2* was detected in 78% (28 of 36) of NPC tissues, whereas all the NP tissues expressed stable *LARS2* mRNA level, indicating that *LARS2* might be involved in the pathogenesis of NPC. Meanwhile, we found that *LARS2* down-regulation showed a significant correlation with lymph node metastasis in NPC patients, implying that the stable expression of *LARS2* may prevent lymph node metastasis of tumor cells in NPC patients to a certain extent.

To assess the possible molecular mechanisms causing *LARS2* inactivation in NPC tissues, we detected the genetic (mainly gene mutation, allelic loss) and epigenetic (mainly promoter methylation) alterations of *LARS2* gene in NPC tissues.

We screened the promoter region and exon 1 of *LARS2* gene for mutations by SSCP and sequencing

analysis in 25 NPC tissues. However, no mutations were found in the two regions in all the tested NPC samples, demonstrating that gene mutation might not be responsible for *LARS2* silencing in NPC. Meanwhile, we analyzed the allelic loss status of *LARS2* in NPC. As LOH or HD can be considered as a reliable indicator for the presence of TSGs in some specific regions with high-frequency LOH or HD [26,27], a number of TSGs have been successfully identified by LOH or HD assay combined with other methods, for example, *sFRP1* and *DFF45* [28,29]. By amplifying RH25266 and SHGC-12886, two microsatellite markers flanking the *LARS2* gene, we found that HD frequency was 28% in NPC specimens at the *LARS2* locus, in accordance with the previously reported high deletion frequency of 3p21.3 in NPC, suggesting that allelic loss may be one of the mechanisms leading to inactivation of *LARS2* in NPC. Nevertheless, more conspicuous down-regulation of *LARS2* detected in NPC strongly suggested that down-regulation of *LARS2* might not be only due to genomic deletions and epigenetic mechanism remained investigated.

Recently, it is recognized that transcriptional silencing by hypermethylation of CpG islands in promoter region has become a very common mechanism for the inactivation of TSGs [30]. Previous studies have demonstrated that the CpG islands in the promoter regions of some TSGs (e.g. *RASSF1A*, *p16*, *LTF*, *DLC1*) are frequently methylated in NPC tissues but are rarely methylated in the corresponding NP tissues. In this study, aberrant DNA methylation of *LARS2* gene was found in 64% of primary NPC tissues but only in 12.5% of NP tissues. Statistical analysis showed that the difference in hypermethylation level of *LARS2* gene between NPC and NP tissues was of statistical significance ($P = 0.017$), implying that promoter methylation may be the major mechanism for inactivation of *LARS2* in NPC. In addition, we found that there may be a correlation between *LARS2* methylation and lymph node metastasis, but further large-scale studies with more NPC samples are necessary and warranted to prove the strength of this contention.

Further parallel analysis demonstrated that hypermethylation of *LARS2* promoter alone could contribute to *LARS2* down-regulation in NPC (e.g. in samples 7, 13, 20, 23) or eliminate *LARS2* expression in NPC combined with HD (e.g. in samples 3, 9, 18), whereas no hypermethylation or HD could keep a normal level of *LARS2* in NPC (e.g. in samples 6, 14, 17, 22) (**Table 2**), also supporting our point of view. However, in two cases with *LARS2* down-regulation (samples 2, 25), no mutation/hypermethylation/HD was detected, indicating that other epigenetic mechanism (e.g. histone deacetylation) may play a certain role in transcriptional silencing of *LARS2* in NPC.

As we know, TSGs could be divided into various categories: classical, e.g. *RB1*, *p53*; haploinsufficient, e.g. $p27^{Kip1}$, *Beclin 1*; cancer-specific or multiple, i.e. involved in several distinct cancers. Unlike the classical TSGs, haploinsufficient TSGs defy the identification through mutation analysis and may be quite common. The genetic and epigenetic alterations of *LARS2* in NPC tissues indicated that *LARS2* might act as a haploinsufficient tumor suppressor in NPC. Meanwhile, the relationship between mitochondrial DNA (mtDNA) and human malignancies attracts much attention in the understanding of carcinogenesis in recent years [31]. For example, metabolic catastrophe in mitochondria can promote death of tumor cells that have disabled apoptosis, and somatic mtDNA alterations including point mutations and microsatellite instability have been frequently detected in human cancers such as prostate cancer and gastric cancer [32,33]. *LARS2* encoding the precursor of mitochondrial leucyl-tRNA synthetase was found to be dramatically down-regulated in NPC in our work, providing us a new insight into further understanding the process of NPC tumorigenesis.

In summary, we have identified that *LARS2* was absent or down-regulated in NPC. Our findings suggested that HD and methylation should play an important role in inactivation of *LARS2* in NPC, but more work is required to be done to elucidate its role in NPC. Upon further study, these unique aberrations will provide insight into mechanisms of NPC carcinogenesis.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (No. 30801322).

References

1 Tao Q and Chan AT. Nasopharyngeal carcinoma: molecular pathogenesis and therapeutic developments. Expert Rev Mol Med 2007, 9: 1–24
2 Wei WI and Sham JS. Nasopharyngeal carcinoma. Lancet 2005, 365: 2041–2054
3 Lo KW, To KF and Huang DP. Focus on nasopharyngeal carcinoma. Cancer Cell 2004, 5: 423–428
4 Huang Z, Desper R, Schaffer AA, Yin Z, Li X and Yao K. Construction of tree models for pathogenesis of nasopharyngeal carcinoma. Genes Chromosomes Cancer 2004, 40: 307–315

5 Yan W, Song L, Wei W, Li A, Liu J and Fang Y. Chromosomal abnormalities associated with neck nodal metastasis in nasopharyngeal carcinoma. Tumour Biol 2005, 26: 306–312

6 Chen YJ, Ko JY, Chen PJ, Shu CH, Hsu MT, Tsai SF and Lin CH. Chromosomal aberrations in nasopharyngeal carcinoma analyzed by comparative genomic hybridization. Genes Chromosomes Cancer 1999, 25: 169–175

7 Fang Y, Guan X, Guo Y, Sham J, Deng M, Liang Q and Li H, *et al*. Analysis of genetic alterations in primary nasopharyngeal carcinoma by comparative genomic hybridization. Genes Chromosomes Cancer 2001, 30: 254–260

8 Hui AB, Or YY, Takano H, Tsang RK, To KF, Guan XY and Sham JS, *et al*. Array-based comparative genomic hybridization analysis identified *cyclin D1* as a target oncogene at 11q13.3 in nasopharyngeal carcinoma. Cancer Res 2005, 65: 8125–8133

9 Chan AS, To KF, Lo KW, Mak KF, Pak E, Chiu B and Tse GM, *et al*. High frequency of chromosome 3p deletion in histologically normal nasopharyngeal epithelia from southern Chinese. Cancer Res 2000, 60: 5365–5370

10 Zhou L, Jiang W, Ren C, Yin Z, Feng X, Liu W and Tao Q, *et al*. Frequent hypermethylation of *RASSF1A* and *TSLC1*, and high viral load of Epstein–Barr virus DNA in nasopharyngeal carcinoma and matched tumor-adjacent tissues. Neoplasia 2005, 7: 809–815

11 Yi HM, Ren CP, Peng D, Zhou L, Li H and Yao KT. Expression, loss of heterozygosity, and methylation of *GNAT1* gene in nasopharyngeal carcinoma. Ai Zheng 2007, 26: 9–14

12 Yi HM, Li H, Peng D, Zhang HJ, Wang L, Zhao M and Yao KT, *et al*. Genetic and epigenetic alterations of *LTF* at 3p21.3 in nasopharyngeal carcinoma. Oncol Res 2006, 16: 261–272

13 Qiu GH, Tan LK, Loh KS, Lim CY, Srivastava G, Tsai ST and Tsao SW, *et al*. The candidate tumor suppressor gene *BLU*, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress-responsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. Oncogene 2004, 23: 4793–4806

14 Kiss H, Kedra D, Yang Y, Kost-Alimova M, Kiss C, O'Brien KP and Fransson I, *et al*. A novel gene containing LIM domains (LIMD1) is located within the common eliminated region 1 (C3CER1) in 3p21.3. Hum Genet 1999, 105: 552–559

15 Sambrool J, Fritsch EF and Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. NY: Cold Spring Harbor Laboratory Press, 1989

16 Senchenko VN, Liu J, Loginov W, Bazov I, Angeloni D, Seryogin Y and Ermilova V, *et al*. Discovery of frequent homozygous deletions in chromosome 3p21.3 LUCA and AP20 regions in renal, lung and breast carcinomas. Oncogene 2004, 23: 5719–5728

17 Peng D, Ren CP, Yi HM, Zhou L, Yang XY, Li H and Yao KT, *et al*. Genetic and epigenetic alterations of *DLC-1*, a candidate tumor suppressor gene, in nasopharyngeal carcinoma. Acta Biochim Biophys Sin 2006, 38: 349–355

18 Zhou L, Feng XL, Shan WJ, Zhou W, Liu W, Wang L and Zhu B, *et al*. Epigenetic and genetic alterations of the *EDNRB* gene in nasopharyngeal carcinoma. Oncology 2007, 72: 357–363

19 Tuhkanen H, Anttila M, Kosma VM, Ylä-Herttuala S, Heinonen S, Kuronen A and Juhola M, *et al*. Genetic alterations in the peritumoral stromal cells of malignant and borderline epithelial ovarian tumors as indicated by allelic imbalance on chromosome 3p. Int J Cancer 2004, 109: 247–252

20 Hesson LB, Cooper WN and Latif F. Evaluation of the 3p21.3 tumour-suppressor gene cluster. Oncogene 2007, 26: 7283–7301

21 Protopopov A, Kashuba V, Zabarovska VI, Muravenko OV, Lerman MI, Klein G and Zabarovsky ER. An integrated physical and gene map of the 3.5-Mb chromosome 3p21.3 (AP20) region implicated in major human epithelial malignancies. Cancer Res 2003, 63: 404–412

22 Kok K, Naylor SL and Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv Cancer Res 1997, 71: 27–92

23 Kwong J, Chow LS, Wong AY, Hung WK, Chung GT, To KF and Chan FL, *et al*. Epigenetic inactivation of the deleted in lung and esophageal cancer 1 gene in nasopharyngeal carcinoma. Genes Chromosomes Cancer 2007, 46: 171–180

24 't Hart LM, Hansen T, Rietveld I, Dekker JM, Nijpels G, Janssen GM and Arp PA, *et al*. Evidence that the mitochondrial leucyl tRNA synthetase (*LARS2*) gene represents a novel type 2 diabetes susceptibility gene. Diabetes 2005, 54: 1892–1895

25 Munakata K, Iwamoto K, Bundo M and Kato T. Mitochondrial DNA 3243A > G mutation and increased expression of *LARS2* gene in the brains of patients with bipolar disorder and schizophrenia. Biol Psychiatry 2005, 57: 525–532

26 Zabarovsky ER, Lerman MI and Minna JD. Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene 2002, 21: 6915–6935

27 Angeloni D. Molecular analysis of deletions in human chromosome 3p21 and the role of resident cancer genes in disease. Brief Funct Genomic Proteomic 2007, 6: 19–39

28 Stoehr R, Wissmann C, Suzuki H, Knuechel R, Krieg RC, Klopocki E and Dahl E, *et al*. Deletions of chromosome 8p and loss of *sFRP1* expression are progression markers of papillary bladder cancer. Lab Invest 2004, 84: 465–478

29 Yang HW, Chen YZ, Piao HY, Takita J, Soeda E and Hayashi Y. DNA fragmentation factor 45 (*DFF45*) gene at 1p36.2 is homozygously deleted and encodes variant transcripts in neuroblastoma cell line. Neoplasia 2001, 3: 165–169

30 Kwong J, Lo KW, To KF, Teo PM, Johnson PJ and Huang DP. Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin Cancer Res 2002, 8: 131–137

31 Modica-Napolitano JS, Kulawiec M and Singh KK. Mitochondria and human cancer. Curr Mol Med 2007; 7: 121–131

32 Jerónimo C, Nomoto S, Caballero OL, Usadel H, Henrique R, Varzim G and Oliveira J, *et al*. Mitochondrial mutations in early stage prostate cancer and bodily fluids. Oncogene 2001, 20: 5195–5198

33 Hiyama T, Tanaka S, Shima H, Kose K, Tuncel H, Ito M and Kitadai Y, *et al*. Somatic mutation in mitochondrial DNA and nuclear microsatellite instability in gastric cancer. Oncol Rep 2003, 10:1837–1841