Original Article

Evaluation of Three Pathogenic Mutations in ALK Gene in Neuroblastoma in Iranian Children

Farnaz Vahidian [1], Fatemeh Pourrajab [2], Seyed Mohsen Miresmaeili [1]*

[1]Department of Biology, Science and Arts University, Yazd, Iran. [2]Assistant professor of Clinical Biochemistry and Molecular Biology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Abstract

Neuroblastoma is one of the most common solid tumors in children which is originated from precursor sympathetic nerves. *ALK* gene has been studied in cellular differentiation and cell migration in neuroblastoma. Recently, three new mutations in the *ALK* gene have been investigated, which can be used as a target for diagnosis. These mutations are recorded with international identifiers rs113994087, rs113994089, and rs281864720. The aim of this study was to investigate these mutations in Iranian patients with neuroblastoma using ARMS PCR technique in order to evaluate these mutations as a diagnostic and prognostic marker. The study was designed as a case-control study. In the case group, 45 children with neuroblastoma, whose pathology confirmed the disease, were placed. In the control group, 27 age-matched children with neuroblastoma were placed. 3 ml of blood sample with EDTA anticoagulant was collected and then DNA extracted from the blood samples evaluated by ARMS PCR method followed by gel electrophoresis.

In this study, the frequency of three pathogenic mutations rs113994087, rs113994089 and rs281864720 in children with neuroblastoma was studied in Iranian population. The average age of the children under study was 6.6 years and gender was almost equal in frequency. The frequency of homozygous mutants for the rs113994087, rs113994089, and rs281864720 mutations were 22.3, 24.6 and 22.3%, respectively, while the frequency of these mutations in the control group was significantly lower. These mutations were studied for the first time in the Iranian population and showed a significant rate of abundance.

Keywords: Neuroblastoma, ALK gene, Mutation, Cancer

Introduction

Neuroblastoma is one of the most common solid tumors in children. These tumors originate from progenitors of sympathetic nerve cells and often develop in adrenal glands [1]. This cancer often affects children and in some cases is detectable at the first month after birth. Unlike other childhood cancers, the survival rate is relatively low (less than 50%), especially in patients with high-risk neuroblastoma [2]. Symptoms include palpable masses in the abdomen, neck or chest, raised eyes, dark circles around the eyes, bone pain, dyspnea, and muscle weakness [3]. Important prognostic factors include children's age and tumor stage at diagnosis [4]. Generally, patients with localized tumors have the best prognosis, while patients with metastatic tumors and those older than two years old have the poorest prognosis [5]. Neuroblastoma is responsible for approximately 8% of childhood cancers. The incidence of neuroblastoma reaches to approximately 650 new cases per year in developed countries; however, the incidence is lower in developing countries of Asia, Latin America and Africa [6]. In terms of mortality, the 5-year survival rates have been 83%, 55%, and 40% in neonates, children of 1 -5 years, and children >5 years old, respectively. Advances in diagnostic brain imaging methods and new therapeutic approaches have improved neuroblastoma therapeutic outcomes in recent years. Although patient's race seems to have no prognostic impact, the incidence of neuroblastoma is more common in white than in black children [7].

In the last two decades, many chromosomal, genetic, and molecular abnormalities have been identified in patients with neuroblastoma. Some of these genetic alterations have been used as diagnostic markers, some as prognostic markers, and some as the basis for formulating new therapeutic strategies [8]. The Anaplastic Lymphoma Receptor Tyrosine Kinase (*ALK*) is one of the most important genes involved in neuroblastoma pathogenesis. Studies suggested *ALK* gene roles in the cellular differentiation and migration of tumor

Address for correspondence: Mohsen MirEsmaeili, Department of Biology, Science and Arts University, Yazd, Iran.
Email: Smm230 @ gmail.com

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cells in neuroblastoma. The *ALK* gene mutations have been identified in 50% of inherited, 8-10% of sporadic, and 100% of high-risk cases of neuroblastoma [9]. The *ALK* gene encodes a tyrosine kinase receptor which belongs to the insulin receptor gene superfamily. The protein product of the *ALK* gene has three extracellular and intracellular domains and a second membrane-bound hydrophobic domain. This protein plays a substantial role in the development of brain and sympathetic neurons of the nervous system. The *ALK* gene is located on the short arm of chromosome 2 (2p23) and has a size of 728 kb. Mutations of the *ALK* gene most commonly occur in the respective kinase domain of the protein. Concurrent *ALK* mutation and *MYC-N* duplication has been reported in neuroblastoma [10].

Nucleotide substitutions at the nucleotide positions of 1174-1275-1245 are among most common mutations of the *ALK* gene accounting for 85% of all mutations in this gene. Recently, three novel mutations in the *ALK* gene have been identified and suggested as potential diagnostic markers. These mutations have been registered with the international IDs of rs113994087, rs113994089, and rs281864720. The aim of this study was to detect these mutations in Iranian children with neuroblastoma using ARMS-PCR technique. We also assessed diagnostic and prognostic values of these mutations.

Materials and Methods

The patients included 45 children diagnosed with neuroblastoma referred to Tabriz International Hospital during 2013-2016. The diagnosis of neuroblastoma had been confirmed by histopathological examination, and all the patients had been undergone surgical resection of the tumors. Exclusion criteria included histories of chemotherapy and radiotherapy in patients with neuroblastoma.

The control group included 27 age-matched children without neuroblastoma referred to the same hospital. After obtaining ethical approval and informed consents from the children's parents, the patients' demographic and clinical information including age, gender, and the stage of disease was recorded. Venous blood samples (3 ml) were obtained from the patients and collected in vacutainer tubes containing K3EDTA anticoagulant. The samples were immediately transferred to the hospital laboratory and kept in freezer (-80 ° C) until using.

DNA Extraction

DNA was extracted from blood samples using DNA Mini kit (Cat number: 51306, QIAamp, Germany) according to the manufacturer's instructions. The quality of the extracted DNA (in terms of size and fragments) was verified using Nanodrap spectrophotometer (Mastogen, USA) and agarose gel electrophoresis. The genetic mutations of rs113994087, rs113994089, and rs281864720 were assessed using ARMS-PCR. Specific primer pairs were designed using Primer 3 software to identify the mutant and normal alleles of these mutations.

Results and Discussion

There were no significant differences in the demographic features between the two groups. (P value=0.3). Demographic features of patients and controls have been shown in **Table 1**.

Table 1. The Demographic Characteristics of Children with Neuroblastoma and Healthy Counterparts

Parameters		Study Groups: Case N= 45 N (%)	Study Groups: Control N=27 N (%)
Mean Age, Months (Range)		80.2 (5-168)	88.5 (10-132)
Gender	Male	24 (53.3)	13 (48.1)
	Female	21 (46.7)	14 (51.9)

The rs113994087 **(Figure 1)**, rs113994089 **(Figure 2)**, and rs281864720 **(Figure 3)** mutations of *ALK* gene were detected by ARMS-PCR and visualized using agarose gel electrophoresis. The distributions of genotypes of *ALK* mutations; (rs113994087, rs113994089, and rs281864720) have been demonstrated in **Table 2**.

Table 2. Genotypes of ALK Mutations; (rs113994087, rs113994089, and rs281864720) in Iranian Children with Neuroblastoma and Healthy Counterparts

Mutations		Study Groups: Case N= 45 n (%)	Study Groups: Control N=27 n (%)
Rs1139940890	**Normal Homozygote**	26 (57.7)	19 (70.3)
	Heterozygote	9 (20)	5 (18.5)
	Mutant Homozygote	10 (22.3)	3 (11.2)
Rs113994720	**Normal Homozygote**	26 (57.7)	19 (70.3)
	Heterozygote	8 (17.7)	5 (18.5)
	Mutant Homozygote	11 (24.6)	3 (11.2)
Rs113994087	**Normal homozygote**	29 (64.4)	21 (77.6)
	Heterozygote	6 (13.3)	3 (11.2)
	Mutant Homozygote	10 (22.3)	3 (11.2)

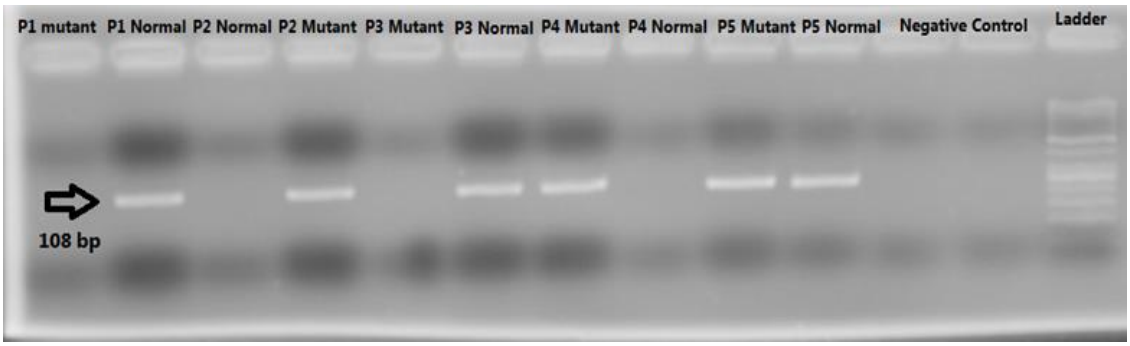

Figure 1. Gel Electrophoresis of rs113994087 Mutation of *ALK* Gene in 5 Children with Neuroblastoma. From left: Patients 1 and 3: Normal Homozygous, Patients 2 and 4: Mutant Homozygous, Patient 5: Heterozygote. DNA Ladder Size: 100bp.

Figure 2. Gel Electrophoresis of rs113994089 Mutation of *ALK* Gene in 5 Children with Neuroblastoma. From Left: Patients 1, 2, and 3: Mutant Homozygous, Patient 4: Normal Homozygous, Patient 5: Heterozygote. DNA Ladder Size: 100bp.

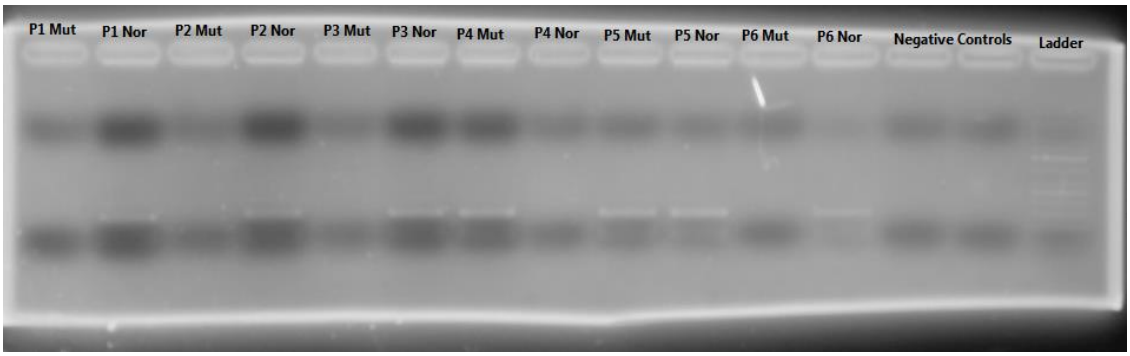

Figure 3. Gel Electrophoresis of rs113994720 Mutation of *ALK* Gene in 6 Children with Neuroblastoma. From Left: Patients 1, 2, 3, and 6: Normal Homozygous, Patient 4: Mutant Homozygous, Patient 5: Heterozygote. DNA Ladder Size: 100bp.

Several mutations of *ALK* gene have been associated with the development of familial and sporadic neuroblastomas. The *ALK* gene encodes a tyrosine kinase receptor. Although the exact function of this protein is unknown, its role in cellular proliferation has been suggested. Mutations in *ALK* gene results in the production of an abnormally and continuously activated tyrosine kinase receptor inducing uncontrolled proliferation of immature neurons which leads to the development of neuroblastoma.

In the present study, the frequencies of three pathogenic mutations within the *ALK* gene (i.e., rs113994087, rs113994089, and rs281864720) were investigated in Iranian children with neuroblastoma. The mean ages of the studied children were 6.68 and 7.37 years with almost equal gender distribution in the case and control groups, respectively.

The frequencies of homozygous mutant genotypes of rs113994087, rs113994089, and rs281864720 mutations in the studied population were 22.3%, 24.6% and 22.3%, respectively. The frequencies of mutant genotypes were significantly lower in the control than case group.

The rs113994087 mutation is resulted from a clinically important missense nucleotide substitution in the *ALK* gene. This mutation can be identified in both somatic and germline cells. Three allelic states of this mutation include A / G / T nucleotides. In this study, we investigated the presence of A and G alleles using ARMS-PCR. The rs113994089 mutation

is also caused by a clinically significant missense mutation in either somatic or germline cells. Three allelic states of this mutation include A / G / C nucleotides. In the present study, the C and G alleles were detected by ARMS PCR. Finally, the rs281864720 mutation is resulted from a clinically important missense mutation in the *ALK* gene in either somatic or germline cells. Using ARMS-PCR, we here investigated the C and G alleles of this polymorphism.

In the present study, the ARMS-PCR method using three primers was applied to detect the mutations of the *ALK* gene. The common reverse primer and specific forward primers were designed to specifically detect each mutation at the last nucleotide of 3 end. This strategy is a cost-effective way requiring a lower number of primers. In another point of view, all the primers designed here had same "Tm" temperatures and annealed at 56 ° C. Therefore, these three polymorphisms can be detected by a multiplex PCR approach which is a cost-effective and time-saving method.

In one study, Berry *et al*. assessed the impact of F1174L mutation of the *ALK* gene on tumor development and its interaction with *MYC-N* oncogene in mouse model of neuroblastoma [11]. The results of the recent study showed that the F1174L mutation played a major pathogenic role in the development and progression of neuroblastoma through inducing the expression of *MYC-N*.

In the study of Lerosey *et al*., somatic mutations (using PCR) and gene duplication (using CGH) of the *ALK* gene were studied in patients' samples and cell lines of neuroblastoma [12]. In the recent study, the *ALK* oncogene was overexpressed in neuroblastoma tumor specimens indicating its role in the development and progression of neuroblastoma. Furthermore, knocking down the mutated *ALK* in a neuroblastoma cell line profoundly decreased cellular proliferation.

In a study in 2008, Chen *et al*. sequenced the *ALK* gene in samples from neuroblastoma patients and cell lines [13]. They revealed that 6.1% of patients' specimens and 33% of neuroblastoma cell lines had missense mutations in the *ALK* gene. Most of the mutations (except for 1 case) occurred in stages 3 and 4 of the disease and within the kinase domain of ALK protein. The mutant kinase domain confers the receptor with self-phosphorylation ability resulting in increased and continuous kinase activity.

Conclusion

The frequencies of the pathogenic mutations studied here were higher in children with neuroblastoma than healthy counterparts. Therefore, these mutations can be used as screening markers for identifying children who are at risk of neuroblastoma. However, as these mutations were not significantly associated with the stage of the disease, they cannot be used as prognostic markers in patients with neuroblastoma.

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