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Evaluation of *KRAS let-7 lcs6* Polymorphism in Korean Populations by Pyrosequencing

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Abstract : A single nucleotide polymorphism (SNP) in the 3′-untranslated region of the *KRAS* oncogene (rs61764370) was recently reported to act as a genetic marker for increased risk of developing human cancers. The frequency of the variant allele is 5.8% in world population, however, there is no study in Korean population with large cases. In present study, we evaluated the *KRAS let-7 lcs6* SNP in Korean population for the first time.

Genotyping was performed in 178 randomized subjects. DNA was extracted and SNP was evaluated by using PCR and pyrosequencing to identify the T or G allele of the *KRAS let-7 lcs6* SNP.

As a result, only T allele was found in all subjects and quantitative sequencing result showed that the proportion of G allele was extremely low.

This study confirmed that *KRAS let-7 lcs6* have only one allele system in Korean population by sensitive and quantitative pyrosequencing for the first time. Its clinical significance should be studied further.

Keywords : *KRAS*, Single nucleotide polymorphism (SNP), *let-7 lcs6*

Introduction

MicroRNAs (miRNA) are small noncoding RNAs that regulate gene expression by degrading and/or suppressing the translation of target messenger RNA (mRNA) by binding to partially complementary sites in the 3′-untranslated region (UTR) of mRNA (Calin et al. 2004, Esquela-Kerscher and Slack 2006, Carletti and Christenson 2009). Recently, miRNAs have been identified as important factors in cancer development and progression because it can act as both tumor suppressors and oncogenes (Johnson et al. 2005, Paranjape et al. 2009, Reddy et al. 2010). As global gene regulators, single nucleotide polymorphisms (SNPs) within miRNAs or miRNA binding sites can affect gene expression by interfering with microRNA and function and, thereby, result in various pathological processes and drug response (Sethupathy and Collins 2008, Yang et al. 2008, Mishra and Bertino 2009, Ratner et al. 2010).

The lethal-7 (*let-7*) was one of the first discovered miRNA families associated with various cancers (Johnson et al. 2005, Chin et al. 2008, Yang et al. 2008). And a SNP (rs61764370) in a *let-7* complementary site (LCS) of the *KRAS* 3′-UTR has been reported to have an increased cancer risk and poor prognosis by increasing *KRAS* expression especially in lung cancers (Christensen et al. 2009, Nelson et al. 2010, Paranjape et al. 2011, Pharoah et al. 2011). On the other hand, recent studies showed that *let-7 lcs6* variant have a better outcome and good response to cetuximab (Smits et al. 2011). The frequency of *KRAS let-7 lcs6* polymorphism was studied in 2,433 samples representing 46 geographic populations by Chin et al. (2008). According to this study, it was only 5.8% of the world population and almost absent in East Asians and Native Americans, was uncommon in Africans. Because only 48 Korean samples

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were analyzed in this study, further study with larger samples was needed to clarify its SNP status in Korean population.

In present article, we reevaluated a *KRAS let-7 lcs6* polymorphism in Korean population for the first time. Because clinical significance of this SNP has been focused increasingly, its frequency in Korean population should be definitively identified. To obtain more accurate result, pyrosequencing method revealing the quantity of the alleles was used.

Materials and Methods

Subjects were selected randomly in Keimyung Human Bio-Resource Bank at Dongsan Medical Center between 1999 and 2003. The investigated population was recruited in the regions of Daegu, which exhibit the similarities in socio-economical conditions. Present study is ethnically homogenous population and therefore suitable for the determination of polymorphism. The institutional regional review board approved the research proposal (IRB No,10-157), and informed consent was obtained from all individuals involved in the study. Genomic DNA was prepared from 178 individuals using DNA absolute extraction kit (Absolute^{TM} DNA extraction Kit, BioSewoom, Korea) according to manufacturer's instructions. Primers for amplification and pyrosequencing were designed using Pyrosequencing Assay Design Software (Biotage AB, Sweden). The primers for polymerase chain reaction (PCR) and pyrosequencing were schematically described in Fig. 1. PCR was performed by using a thermal cycler (Applied Biosystems, USA) in the order as follows: 40 cycles of 30 sec at 94˚C for denaturation, 30 sec at 55˚C for annealing, and 60 sec at 72˚C for extension. Final extension was performed at 72˚C for 10 min. The pyrosequencing reaction was performed on a PyroMark Q24 instrument using the Pyro Gold Q24 Reagents (Qiagen, Netherlands). The pyrosequencing primers were used in a final concentration of 0.3 μmol/L. Resulting data were analyzed and quantified with the PyroMark Q24 software version 2.0 (Qiagen, Netherlands).

SPSS 18.0 software for Windows (Chicago, USA) was used. Chi-square, Fischer' exact tests, and Student' t-test were used to analyze the relationship between variables. A p value < 0.05 was considered statistically significant.

Results

One hundred and seventy eight subjects were randomly selected and their lifestyle habits and possible exposure-related effects were recorded in detailed questionnaires and considered in the statistical analyses. There were no significant differences between age and sex distribution, suggesting that the matching based on these 2 variables was adequate. The characteristics of participant were summarized in Table 1. The PCR products were electrophoresed on an agarose gel to confirm the size of the bands (137 bp), and then, pyrosequencing was performed. The result of pyrosequencing showed that *let-7 lcs6* polymorphism had only T allele in all Korean populations (Fig. 2). According to the quantitative measurement, proportion of mutated

Table 1. The characteristic of the participant in present study

	Total number (%)	Male (N, %)	Female (N, %)
Age*	64.49±12.43	63.73±13.45	65.87±10.27
≤ 50	24 (13.5)	17 (14.7)	7 (11.3)
51~60	32 (18.0)	20 (17.2)	12 (19.4)
61~70	66 (37.1)	45 (38.8)	21 (33.9)
71~80	46 (25.8)	25 (21.6)	21 (33.9)
≥81	10 (5.6)	9 (7.8)	1 (1.6)
Total	178 (100)	116 (100)	62 (100)

*: years, Mean±SD.

Fig. 1. Pyrosequencing assay design. PCR amplifies a 137-bp fragment containing *KRAS let-7 lcs6*. The sequencing primer can detect polymorphism site (T/G).

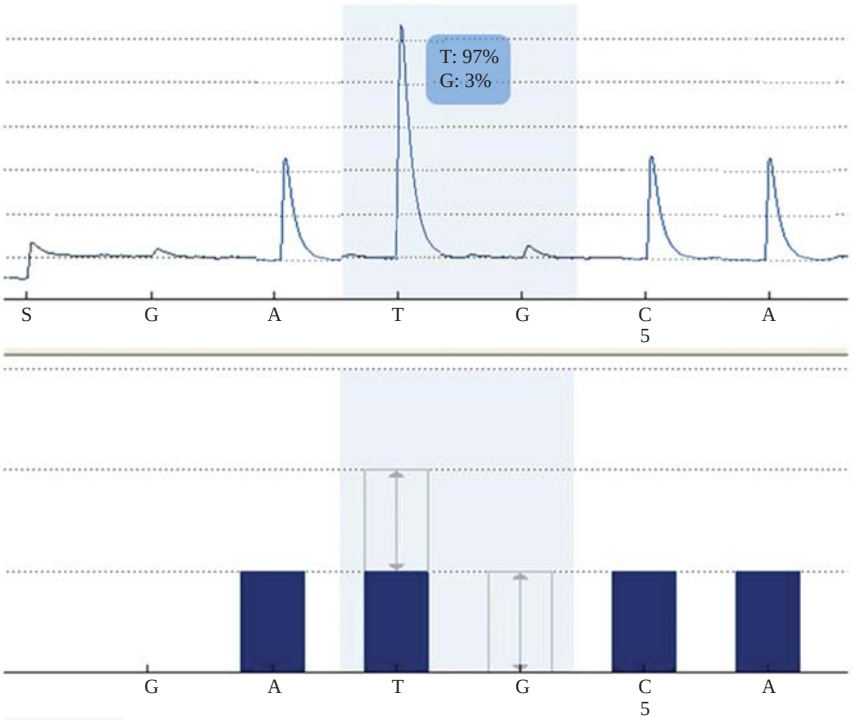

Fig. 2. Representative result of pyrosequencing analysis for *KRAS let-7 lcs6* polymorphism in Korean population. All subjects showed a wild type (T allele) in this site and G allele was extremely low.

type DNA (G allele) was extremely low from 2% to 8%.

Discussion

This is the first evaluation of a *let-7 lcs6* polymorphism in Korean population. Though the recent attention for *let-7* has been increased because of clinical importance of miRNAs, its polymorphism has not been mentioned and studied in Korean population. Chin et al. (2008) studied the frequency of *KRAS let-7 lcs6* polymorphism in various populations and East Asians including Koreans, Oceanians, and Americans showed only T allele. However, Europeans and some Asians with G allele were found in about 10% of the populations. Considering the spread of mankind and genetic association between Asians, we could not rule out the possibility of the presence of G allele in Korean population (Yoshiura et al. 2006, Martínez-Cruz et al. 2011, Peng et al. 2011). So, present study reevaluated a *KRAS let-7 lcs6* polymorphism in Korean populations using pyrosequencing.

As a result, all participants showed wild-type TT genotype regardless gender and age. This result was in agreement with Chin et al. (2008) and it proves that there is only one allele type in Korean population. *KRAS let-7 lcs6* genotype had cancer-related clinical significance according to previous studies (Chin et al. 2008, Christensen et al. 2009, Nelson et al. 2010, Ratner et al. 2010, Paranjape et al. 2011, Pharoah et al. 2011, Smits et al. 2011). Ratner et al. (2010) described that T/G or G/G genotype (G-allele) was associated with ovarian cancer risk and Chin et al. (2008) presented that a SNP in this site increases non-small cell lung cancer risk. In breast and oral cancers, *let-7 lcs6* polymorphism was also associated poor prognosis or cancer risk (Christensen et al. 2009, Paranjape et al. 2011). On the other hand, Nelson et al. (2010) presented no association between *KRAS let-7 lcs6* polymorphism and non-small cell lung cancer. Moreover, the patient with *KRAS let-7 lcs6* variant showed a good prognosis in early-stage colorectal cancer (Smits et al. 2011). However, it is confirmed that variant type was associated with increased transcription and translation of *KRAS*, increasing cell survival and proliferation by the effects of Ras activation (Schubbert et al. 2007). Though the hypothesis that the *KRAS*-

variant increases the risk of developing various cancers is controversial, Korean population with wild type entirely may have low possibility of cancer development than the populations with T/G or G/G genotype.

Pyrosequencing is a quantitative sequencing method based on real-time monitoring of DNA synthesis by measuring the incorporation of each of the four nucleotides at each template position in an automated process (Ronaghi et al. 1998, Ramon et al. 2003, Ahmadian et al. 2006). So, the amount of each template present can be calculated with greater sensitivity and this study showed extremely low proportion of variant allele in *let-7 lcs6* site. Therefore, this article confirmed that Korean population has only wild type of *KRAS let-7 lcs6* for the first time by using pyrosequencing.

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파이로시퀀싱을 이용한 한국인에서의 *KRAS let-7 lcs6* 유전자의 단일염기다형성 분석

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간추림 : *KRAS* 유전자의 3′-비해석부위의 단일염기다형성(rs61764370)은 암발생을 증가시키는 중요한 유전학적 표지자라고 보고되었다. 전세계적으로 이 부분의 다형성은 5.8%에서 나타나지만 한국인에서는 제대로 연구가 이루어지지 않았다. 따라서 본 연구에서 한국인에서 *KRAS let-7 lcs6* 유전자의 단일염기다형성을 분석해보도록 하겠다.

178명의 무작위로 추출된 한국인의 DNA를 이용하여 중합효소연쇄반응과 파이로시퀀싱을 해보았다. *KRAS let-7 lcs6* 다형성의 T 또는 G 유전자를 확인하기 위해서 파이로시퀀싱 연구를 설계하였다.

그 결과 모든 사람에서 T 유전자만이 관찰되었다. 또한 정량적 분석 결과, G 유전자는 매우 낮게 나타났다.

이 연구의 결과는 파이로시퀀싱을 이용하여 정량적 분석을 통해 한국인에서는 *KRAS let-7 lcs6* 유전자가 다형성을 보이지 않고 오직 T 유전자만 나타나는 형태를 가진다는 것을 처음으로 확인하였다. 이에 대한 임상적 의의에 대해서 더욱 많은 연구가 필요할 것으로 생각된다.

찾아보기 낱말 : *KRAS*, 단일염기다형성 (SNP), *let-7 lcs6*

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