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Peptide nucleic acid-probe based analysis to detect filaggrin mutations in atopic dermatitis patients

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Abbreviations

AD, Atopic Dermatitis; FLG, Filaggrin; PNA, Peptide Nucleic Acid;
WT, wild-type; T_m, melting temperature

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease whose prevalence is increasing worldwide. Filaggrin (FLG) is essential for the development of the skin barrier, and its genetic mutations are major predisposing factors for AD. In this study, we developed a convenient and practical method to detect *FLG* mutations in AD patients using peptide nucleic acid (PNA) probes labeled with fluorescent markers for rapid analysis. Fluorescence melting curve analysis (FMCA) precisely identified *FLG* mutations based on the distinct difference in the melting temperatures of the wild-type and mutant allele. Moreover, PNA probe-based FMCA easily and accurately verified patient samples with both heterozygote and homozygote *FLG* mutations, providing a high-throughput method to reliably screen AD patients. Our method provides a convenient, rapid and accurate diagnostic tool to identify potential AD patients allowing for early preventive treatment, leading to lower incidence rates of AD, and reducing total healthcare expenses.

BACKGROUND

The skin plays a crucial primary defensive role in the body, functioning as a barrier to protect the body from the external environment. The loss of barrier function leads to defective skin homeostasis and finally inflammation. Filaggrin (FLG) is a major epidermal structural protein expressed in the stratum corneum and is essential for the skin barrier function. Palmer *et al.*

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first demonstrated that *FLG* mutations are a predisposing factor for AD in the European population (1). Subsequently, it has been reported that approximately 25% of AD patients in Japan have *FLG* mutations (2, 3), and *FLG* mutations were also found in AD patients in several other Asian countries including South Korea and China (4-6). Over 1000 studies on *FLG* have been published so far, and the correlation between *FLG* mutations and inflammatory diseases has been well documented in recent years, further emphasizing the role of *FLG* in maintaining the skin barrier function (7-9). One of the most interesting findings is that the *FLG* mutation sites vary considerably depending on race and geographic region (4). Only the R501X and E2422X mutations were found in both Europeans and Asians. In Asian countries including South Korea, Japan, China, Singapore and Taiwan, only the 3321delA mutation is commonly detected in AD patients. Other *FLG* mutations including S2554X, S2889X, S3296X, and K4022X mutations have been detected in a high proportion of AD patients in Japan (2). In South Korea, all the mutations except for the S2554X have been detected in AD patients with the 3321delA and K4022X mutations most frequently detected according to recent reports (6).

Peptide nucleic acids (PNAs) are artificial DNA analogues can hybridize to their complementary DNA targets (10). A PNA–DNA hybridization is more sensitive to base mismatches than a typical DNA–DNA hybridization (11), and PNA oligomerization clearly shows a distinct difference in the melting temperatures (T_m) between perfect and mismatch DNA sequences (12). Accordingly, unlike the TaqMan assay used in previous studies (13, 14), only one PNA probe can be used to distinguish the wild-type (WT) allele from the mutant allele based on differences in T_m . Therefore, PNA probes have been widely used in medical diagnostic applications to detect point mutations or SNPs in DNA sequences (15-18).

QUESTIONS ADDRESSED

Since the correlation between AD and *FLG* mutations has been clearly established, there is a need to quickly and accurately identify *FLG* mutations in potential AD patients. The conventional Sanger sequencing technique currently used to detect mutations is severely limited in the number of samples that can be simultaneously and precisely processed. In addition, heterozygote mutations analyzed using the Sanger method can yield differing results depending on the peak size of the sequencing profile. Also, the enormous third *FLG* exon, where mutations are dominantly detected, contains at least 10 repeated units with similar sequences making it more difficult to detect mutations using the Sanger method.

In order to overcome these problems, we developed a rapid and accurate method to detect *FLG* mutations using PNA probes and assessed its application in the medical diagnosis of AD patients.

EXPERIMENTAL DESIGN

All clinical studies were approved by the Institutional Review Boards of Gangnam Severance Hospital (IRB No. 3-2014-0027) and Yonsei University Wonju College of Medicine (IRB No. 2011-84). Genomic DNA was isolated from the peripheral blood of healthy individuals and AD patients, and PCR reactions were performed as previously described (3, 19, 20) to obtain DNA fragments corresponding to five *FLG* mutations, 3321delA, S2554X, S2889X, S3296X or K4022X. Since patient samples with S2554X mutation were not available in this study, we constructed artificial plasmids containing the S2554X mutation. Original PCR products were obtained from the genomic DNA of normal individuals and were cloned into the TA cloning vector (Promega). The S2554X mutation was then inserted into the TA plasmid constructs using the QuikChange II XL site-directed mutagenesis kit (Agilent). The PCR fragments were sequenced using the Sanger method to verify the five *FLG* mutations (Figure 1A).

In this study, PNAs were synthesized as probes to detect five *FLG* mutations (Table S1). In order to easily detect mutations, each probe was designed so that its sequence is a perfect match to the WT allele with at least a 5°C difference in the T_m value between the WT and mutant allele. In addition, the probes were labeled with fluorescent markers, and fluorescence melting curve analysis (FMCA) based on the hybridization method was completed to evaluate T_m values in real time by using the CFX96™ system (BIO-RAD) (Table 1). PCR was performed by using 5 pM of each PNA probe in the reaction mixture, and then the fluorescence intensity of the PNA probe was measured from 40°C to 80°C at intervals of 0.5°C to obtain T_m values.

RESULTS

Figure 1B shows the PNA probe-based fluorescence melting curve analysis (FMCA) for the *FLG* mutations. Since the sequence of the WT allele is a perfect match with that of the probe, the affinity of the WT allele to the PNA probe is stronger than that of the mutant allele, corresponding to the right peak curve showing a high T_m value. In comparison, the mutant allele has a weaker affinity for the probe showing a lower T_m value corresponding to the left peak curve. Thus, the PNA probe-based FMCA precisely and easily detects *FLG* mutations as illustrated by the clear difference in the T_m value between the WT and mutant allele (Figure 1B and Table 1).

In case of normal healthy individuals with no *FLG* mutation, the PNA probe-based FMCA displays the blue-colored melting curves corresponding to high T_m values of the WT allele (Figure 1B). Among the AD patient samples, we detected 3321delA, S2889X, S3296X and K4022X heterozygote mutations. Therefore, two peak curves were detected in the melting curve analysis: the first represents the mutant allele, and the second indicates the WT allele (Figure 1B, green curves). As described above, we also constructed the artificial

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plasmids containing S2554X mutation, and then verified whether our synthesized PNA probe could detect the mutation sites in the artificial plasmids instead of patient samples. As shown in the red-colored melting curves in Figure 1B, all curves displayed only low T_m values of the mutant allele which did not overlap with the blue-colored WT curves. Therefore, these results proved that our method can clearly distinguish between heterozygote and homozygote *FLG* mutations without any additional equipment or computer program, making its application in the medical diagnosis of AD patients easier and cost effective.

In order to verify the accuracy of our PNA method, we performed PNA probe-based FMCA in a cohort of AD patients who were recruited for a previous study (6). Only the genomic DNA and corresponding sample numbers were received, and analyses were conducted without knowing which AD patient samples had *FLG* mutations. As a result, six patients with a 3321delA mutation, one with S2889X, two with S3296X, and three with K4022X were detected among seventy AD patients (Table S2 and Figure S1). These results were consistent with the previous study (6).

CONCLUSIONS

Since the prevalence of AD is steadily increasing worldwide, the development an accurate and specific diagnostic method is in high demand (21). A number of high-throughput methods to detect *FLG* mutations such as the TaqMan assay and mass spectrometry have been suggested (13, 14, 22, 23). Mass spectrometry is an accuracy method that directly analyzes the sample without the use of radioactive or fluorescent labels, but it is very cost prohibitive (24). The validity and efficiency of the TaqMan assay has been verified in the high-throughput screening of *FLG* mutations (13, 25, 26). However, the TaqMan assay needs a different probe for each unique target sequence, one for the WT allele and another for the mutant allele, in order to detect a heterozygote mutation.

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In this study, we applied PNA technology to develop a high-throughput screening of *FLG* mutations in AD patients. Unlike the TaqMan assay, our PNA method needs only one probe to distinguish the mutant allele from the WT allele since the strength of a PNA–DNA hybridization is stronger than that of a typical DNA–DNA hybridization. Our results verify that PNA probe-based fluorescence melting curve analysis is a powerful tool to detect *FLG* mutations and can precisely detect both heterozygote and homozygote *FLG* mutations. Therefore, our method provides a convenient, rapid and accurate diagnosis for potential AD patients allowing for early, personalized medical therapy, thus lowering the incidence rates of AD and reducing total healthcare expenses.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTION

Hwang J designed and performed the experiments, analyzed the data, and wrote the manuscript. Lee S performed the experiments, analyzed the data, and wrote the manuscript. Kim D, Mun J, and Han G assisted in the experiments and analyze the data. Soung NK, Cha-Molstad H, Lee KH, Ryoo IJ, Ahn MJ, and Kim ST supported the preparation of materials and samples. Lee MJ, Yoo YD, Lee HG, and Hong JT assisted in designing the research. Kim H, Choi EH, and Kim SC supported the preparation of clinical samples. Kim BY, Ahn JS, Kwon YT, and Kim SC supervised the research study and revised the manuscript.

Keywords: Atopic Dermatitis, Filaggrin Mutation, Peptide Nucleic Acid (PNA), Medical Diagnosis

REFERENCES

1. Palmer C N A, Irvine A D, Terron-Kwiatkowski A, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature Genetics* 2006; 38: 441.
2. Osawa R, Konno S, Akiyama M, et al. Japanese-specific filaggrin gene mutations in Japanese patients suffering from atopic eczema and asthma. *J Invest Dermatol* 2010; 130: 2834-2836.
3. Nemoto-Hasebe I, Akiyama M, Nomura T, et al. FLG mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema. *Br J Dermatol* 2009; 161: 1387-1390.
4. Osawa R, Akiyama M, Shimizu H. Filaggrin gene defects and the risk of developing allergic disorders. *Allergol Int* 2011; 60: 1-9.
5. Jung M, Choi J, Lee S-A, et al. Pyrrolidone carboxylic acid levels or caspase-14 expression in the corneocytes of lesional skin correlates with clinical severity, skin barrier function and lesional inflammation in atopic dermatitis. *Journal of Dermatological Science* 2014; 76: 231-239.
6. On H R, Lee S E, Kim S E, et al. Filaggrin Mutation in Korean Patients with Atopic Dermatitis. *Yonsei Med J* 2017; 58: 395-400.

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7. McGrath J A, Uitto J. The filaggrin story: novel insights into skin-barrier function and disease. *Trends Mol Med* 2008; 14: 20-27.
8. Irvine A D, McLean W H I, Leung D Y M. Filaggrin Mutations Associated with Skin and Allergic Diseases. *New England Journal of Medicine* 2011; 365: 1315-1327.
9. McAleer M A, Irvine A D. The multifunctional role of filaggrin in allergic skin disease. *J Allergy Clin Immunol* 2013; 131: 280-291.
10. Nielsen P E, Egholm M, Berg R H, et al. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 1991; 254: 1497-1500.
11. Park H, Germini A, Sforza S, et al. Kinetic and affinity analyses of hybridization reactions between peptide nucleic acid probes and DNA targets using surface plasmon field-enhanced fluorescence spectroscopy. *Biointerphases* 2006; 1: 113-122.
12. Ratilainen T, Holmen A, Tuite E, et al. Thermodynamics of sequence-specific binding of PNA to DNA. *Biochemistry* 2000; 39: 7781-7791.
13. Kono M, Nomura T, Ohguchi Y, et al. Comprehensive screening for a complete set of Japanese-population-specific filaggrin gene mutations. *Allergy* 2014; 69: 537-540.
14. Sandilands A, Terron-Kwiatkowski A, Hull P R, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 2007; 39: 650-654.
15. Chen T L, Chang J W, Hsieh J J, et al. A Sensitive Peptide Nucleic Acid Probe Assay for Detection of BRAF V600 Mutations in Melanoma. *Cancer Genomics Proteomics* 2016; 13: 381-386.
16. Itonaga M, Matsuzaki I, Warigaya K, et al. Novel Methodology for Rapid Detection of KRAS Mutation Using PNA-LNA Mediated Loop-Mediated Isothermal Amplification. *PLoS One* 2016; 11: e0151654.
17. D'Agata R, Giuffrida M C, Spoto G. Peptide Nucleic Acid-Based Biosensors for Cancer Diagnosis. *Molecules* 2017; 22.
18. Kim C G, Shim H S, Hong M H, et al. Detection of activating and acquired resistant mutation in plasma from EGFR-mutated NSCLC patients by peptide nucleic acid (PNA) clamping-assisted fluorescence melting curve analysis. *Oncotarget* 2017; 8: 65111-65122.
19. Nomura T, Sandilands A, Akiyama M, et al. Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. *J Allergy Clin Immunol* 2007; 119: 434-440.
20. Nomura T, Akiyama M, Sandilands A, et al. Specific filaggrin mutations cause ichthyosis vulgaris and are significantly associated with atopic dermatitis in Japan. *J Invest*

Dermatol 2008; 128: 1436-1441.

21. McGrath J A. The Molecular Revolution in Cutaneous Biology: Era of Molecular Diagnostics for Inherited Skin Diseases. *J Invest Dermatol* 2017; 137: e83-e86.
22. Ballardini N, Kull I, Soderhall C, et al. Eczema severity in preadolescent children and its relation to sex, filaggrin mutations, asthma, rhinitis, aggravating factors and topical treatment: a report from the BAMSE birth cohort. *Br J Dermatol* 2013; 168: 588-594.
23. Thyssen J P, Thuesen B, Huth C, et al. Skin barrier abnormality caused by filaggrin (FLG) mutations is associated with increased serum 25-hydroxyvitamin D concentrations. *J Allergy Clin Immunol* 2012; 130: 1204-1207 e1202.
24. Jackson P E, Scholl P F, Groopman J D. Mass spectrometry for genotyping: an emerging tool for molecular medicine. *Mol Med Today* 2000; 6: 271-276.
25. Margolis D J, Apter A J, Mitra N, et al. Reliability and validity of genotyping filaggrin null mutations. *J Dermatol Sci* 2013; 70: 67-68.
26. Kono M, Akiyama M, Inoue Y, et al. Filaggrin gene mutations may influence the persistence of food allergies in Japanese primary school children. *Br J Dermatol* 2018.

TABLE

Table 1. Difference in melting temperature (T_m) of PNA probe

		3321delA	S2554X	S2889X	S3296X	K4022X
WT (normal)	mismatch (bp)	0	0	0	0	0
	T _m (°C)	71	62	66	69	54
Mutant	mismatch (bp)	1del	1	2	1	1
	T _m (°C)	60	52.5	36	55	46.5

Table 1 shows the correlation between the number of mismatched base pairs and the T_m value. Since the sequence of the probe is a perfect match for the wild-type (WT) allele, the T_m value is higher than that of the mutant, indicating that the affinity of the PNA probe for the WT allele is stronger than that of the mutant. Del: deletion.

FIGURE LEGENDS

Figure 1. Results from Sanger sequencing method vs PNA probe-based fluorescence melting curve analysis (FMCA)

A. The sequencing results, normal (top) and each *FLG* mutations 3321delA, S2554X, S2889X, S3296X, and K4022X (bottom). The symbols used are as follows: R = A, G; S = C, G; M = A, C; W = A, T.

B. The results of the PNA probe-based fluorescence melting curve analysis demonstrates a clear difference between the T_m values of wild-type (WT) and mutant *FLG* alleles, showing readily distinguishable peak curves; the left peak with a low T_m value represents a mutant allele and the right peak with a high T_m value indicates a WT allele. The blue peak curve represents the normal sample having only WT alleles, the two green peak curves indicate a heterozygote mutation having both WT and mutant alleles, and the red peak curve shows the homozygote mutation having only mutant alleles.

Figure S1. PNA probe-based fluorescence melting curve analysis (FMCA) in a cohort of AD patients who were recruited for a previous study (6)

Six patients with a 3321delA mutation, one with S2889X, two with S3296X and three with K4022X were detected among seventy AD patients, and these results was consistent with the previous study (6).

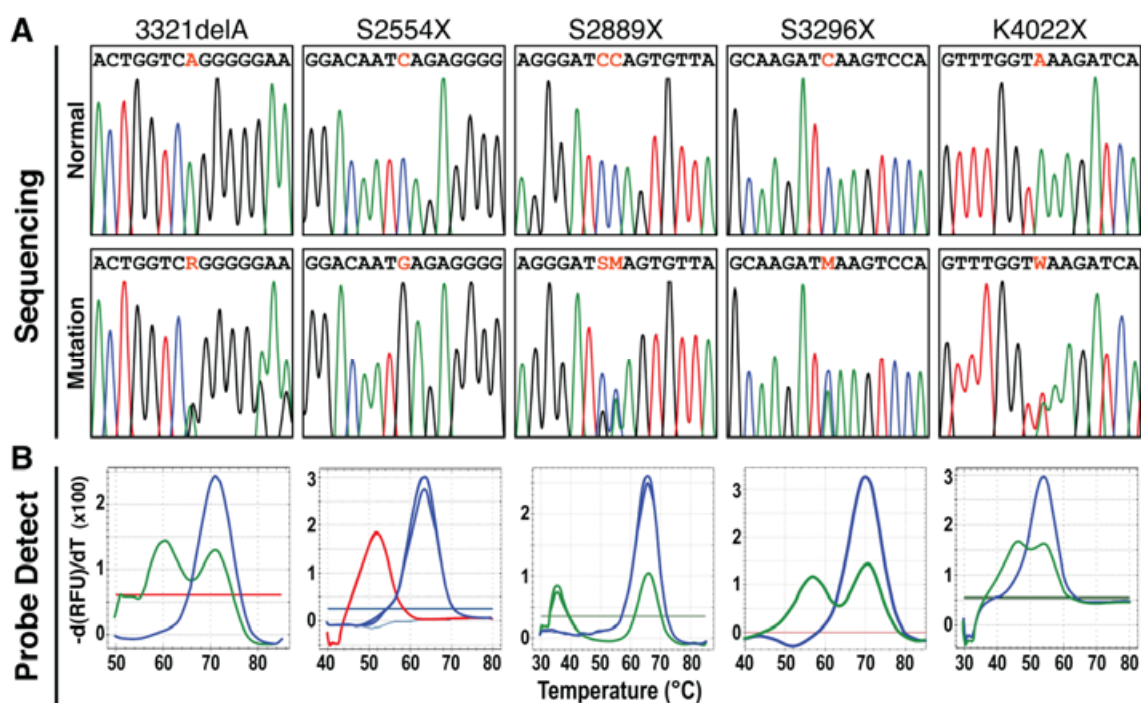
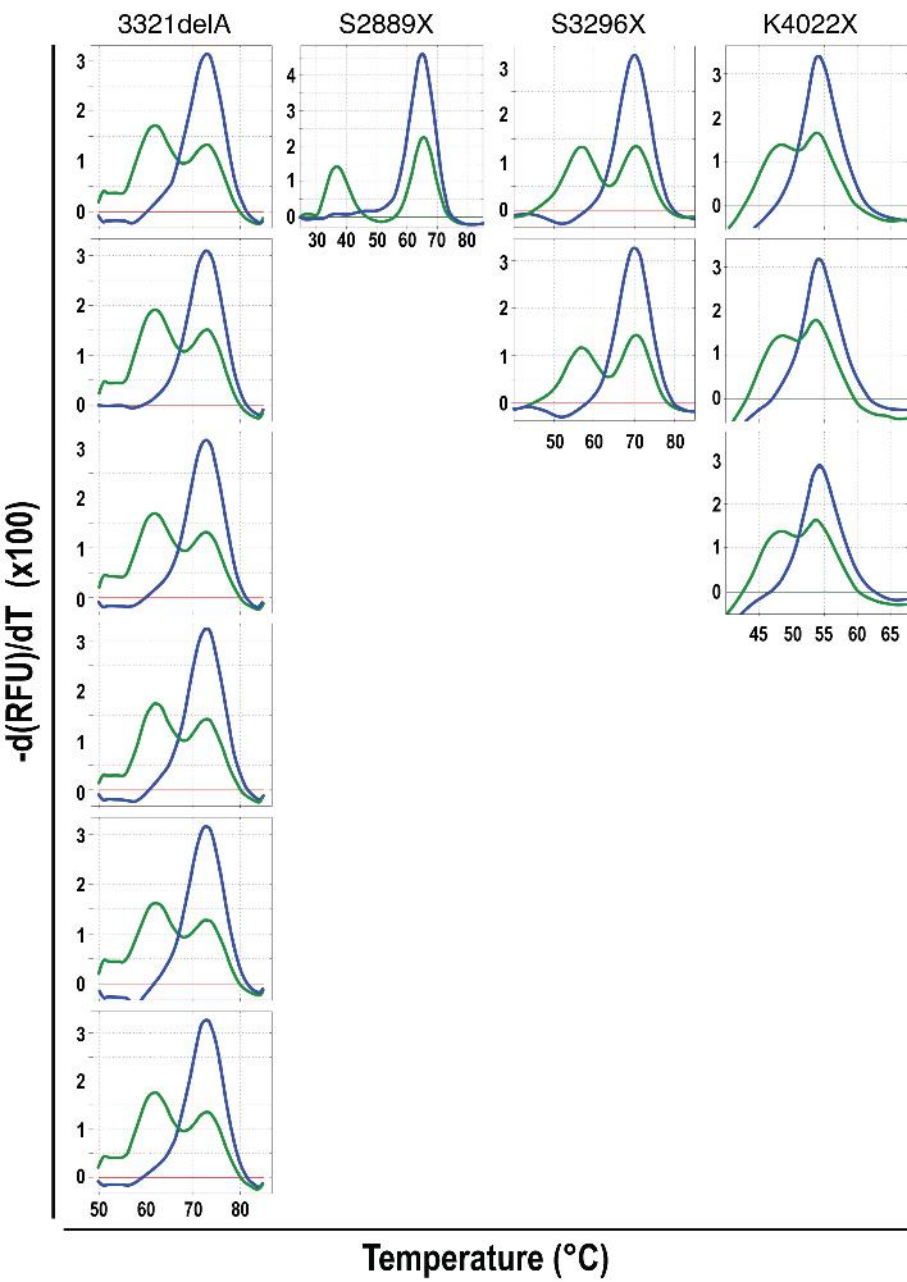


Figure S1



SUPPORTING INFORMATION

Table S1. Synthesized PNA probe for each *FLG* mutation

<i>FLG</i> mutation	Sequence (fluorescent)
3321delA	Dabcyl-GACCTTCCCCCTGACC-O-K (HEX)
S2554X	Dabcyl-CCCCTCTGATTGTCC-O-K (FAM)
S2889X	Dabcyl-ACTGGATCCCTGG-O-K (HEX)
S3296X	Dabcyl-GCAAGATCAAGTCCA-O-K (Texas Red)
K4022X	Dabcyl-GATCTTTACCAAAC-O-K (FAM)

O indicates the linker, and K is the abbreviation for Lysine.

Table S2. PNA probe-based fluorescence melting curve analysis (FMCA) in a cohort of AD patients

Genotype	3321delA	S2554X	S2889X	S3296X	K4022X
AA	64	70	69	68	67
Aa	6	0	1	2	3
aa	0	0	0	0	0
Total	70	70	70	70	70