CHAPTER III

MATERIALS AND METHODS

Research Instrument

1. Pipette tip : 10 μl, 1,000 μl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-rad, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
8. Glass pipette : 5 ml, 10 ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Plastic wrap
13. Stirring-magnetic bar
14. Combs
15. Electrophoresis chamber set
16. Mini-protein 3 electrophoresis
17. Automatic adjustable micropipette : P2 (0.1-2 μl), P10 (0.5-10 μl), P20 (5-20 μl), P100 (20-100 μl), P1000 (0.1-1 ml) (Gilson, France)

1. Pipette boy (Tecnomara, Switzerland)
2. Vortex (Scientific Industry, USA)
3. pH meter (Eutech Cybernatics)
4. Stirring hot plate (Bamstead/Thermolyne, USA)
5. Balance (Precisa, Switzerland)
6. Microcentrifuge (Fotodyne, USA)
7. DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)
8. Thermal cycler (Touch Down, Hybraid USA)
9. Power supply model 250 (Gibco BRL, Scothland)
10. Power poc 3000 (Bio-Rad)
11. Horizon 11-14 (Gibco BRL, Scothland)
12. Sequi-gen sequencing cell (Bio-Rad)
13. Beta shield (C.B.S scientific. Co.)
14. Heat block (Bockel)
15. Incubator (Memmert)
16. Thermostat shaking-water bath (Heto, Denmark)
17. Spectronic spectrophotometers (Genesys5, Milon Roy USA)
18. UV Transilluminator (Fotodyne USA)
19. UV-absorbing face shield (Spectronic, USA)
20. Gel doc 1000 (Bio-RAD)

21. Mitsubishi Video copy processor

22. Thermal paper

23. Refrigerator 4 ^{0}C (Misubishi, Japan)

24. Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

25. Water purification equipment (Water pro Ps, Labconco USA)

26. Water bath

27. Storm 840 and ImageQuaNT solfware (Molecular dynamics)

Reagent

1.General reagent

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular glade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Ficoll 400 (Pharmacia)

1.9 Hydrochloric acid (Merck)

1.10 Mineral oil (Sigma)

1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Sodium chloride (Merck)

1.15 Sodium dodecyl sulfate (Sigma)

1.16 Sodium hydroxide (Merck)

1.17 Sucrose (BDH)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

1.21 40%acrylamide/bis solution 19:1 (Bio-Rad)

1.22 Sigmacote (Sigma)

1.23 Ammoniumperoxodisulfat (Merk)

1.24 [γ^{32}P] ATP (Amershamphamacia Biotech)

1.25 QIAquick PCR purification kit (QIAGEN)

2.Reagent of PCR

2.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (GibcoBRL, Perkin Elmer)

2.2 Magnesium chloride (GibcoBRL, Perkin Elmer)

2.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.4 Oligonucleotide primers (BSU, GENSET) in appendix B

2.5 *Taq* DNA polymerase (GibcoBRL,Perkin Elmer)

2.6 *Taq* Gold DNA polymerase (Perkin Elmer)

2.7 Genomic DNA sample

3.Restriction enzyme

3.1 *HgaI* and 10XNE buffer 1(Biolabs)

3.2 *MspI* and 10XNE buffer 2 (Biolabs)

3.3 T4 kinase and 10Xbuffer (Biolabs)

Procedure

1.Subjects and Sample collection

We obtained 222 blood specimens of case from NPC patients in King Chulalongkorn Memorial Hospital and 368 control samples from blood donating volunteer in Thai Red Cross Society. These patients were interviewed and recorded by questionair. In addition, they were divided into 3 groups on the basis of the grandparent 's race.

Group 1 Thai race were consisted of 139 cases and 124 controls.

Group 2 Chinese race were consisted of 56 cases and 123 controls.

Group 3 Thai-Chinese race were consisted of 27 cases and 121 controls.

2.DNA Extraction

The extraction of DNA from peripheral blood leukocyte was performed as follow:

1. 5-10 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.
2. Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

3. Centrifuge for 8 minutes at 1,000 g, then remove supernatant.

4. Add 3 ml. Cold lysis buffer1, mix thoroughly and centrifuge for 8 minute at 1,000g,

5. Discard supernatant afterward add 900µl lysis buffer2, 10µl

   Proteinase K solution (20 mg Proteinase K in 1.0 ml. Of 1% SDS-

   2mM EDTA, should be prepare 30 min before use.), and 10% SDS 50µl. Mix vigorously for 15 seconds.

6. Incubate the tube(s) in 37°C shaking water bath overnight for complete digestion.

7. Add 1 ml. Phenol-chloroform-isoamyl alcohol shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

8. Transfer the supernatant from each tube (contains DNA) to a new microcentrifuge tube.

9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100%

   ethanol mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dried the pellet. (It is important to rinse well to remove any residual salt and phenol.)

11. Resuspend the digested DNA in 20-300µl of the double distilled water at 37°C until dissolved.

3.Mutation finding by DNA sequencing

To find other mutation on *PIRG* gene, 8 NPC patients were chosen to sequencing for 11 exon of *PIRG*.(except exon 6)

3.1 PCR Amplification for sequencing preparation.

3.1.1 PIGR exon 1

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer	10X	1X	10
$MgCl_2$	25 mM	1.5 mM	6
Taq (Promega)	5 U/μl	2 U/100μl	0.4
Primer Ex-1Fy	10 μM	0.1 μM	1
Primer Ex-1Ry	10 μM	0.1 μM	1
DNA	50 ng/μl	100 ng	10
dH_2O			69.6
total			100

2. PCR condition

95° 4 minutes

95° 1 minute , 55° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

3.1.2 PIGR exon 2

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer	10X	1X	10
$MgCl_2$	25 mM	1.5 mM	6
Taq (Promega)	5 U/μl	2 U/100μl	0.4
Primer Ex-2Fy	10 μM	0.1 μM	1
Primer Ex-2Ry	10 μM	0.1 μM	1
DNA	50 ng/μl	100 ng	10
dH_2O			69.6
total			100

2. PCR condition

95° 4 minutes

95° 1 minute , 55° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

3.1.3 PIGR exon 3

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2

	Strock concentration	Working concentration	using(μl)
10Xbuffer($MgCl_2$)	10X	1X	10
Taq hot start (QIAGEN)	5 U/μl	2 U/100μl	0.4
Primer Ex-3Fy	10 μM	0.3 μM	3
Primer Ex-3Ry	10 μM	0.3 μM	3
DNA	50 ng/μl	200 ng	15
dH_2O			66.6
total			100

2. PCR condition

95° 15 minutes

95° 1 minute , 60° 1 minute , 72° 2 minute 35 cycles

72° 7 minute

3.1.4 PIGR exon 4

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer($MgCl_2$)	10X	1X	10
Taq hot start (QIAGEN)	5 U/μl	2 U/100μl	0.4
Primer Ex-4Fy	10 μM	0.4 μM	4

	Strock concentration	Working concentration	using(μl)
Primer Ex-4Ry	10 μM	0.4 μM	4
DNA	50 ng/μl	200 ng	30
dH_2O			49.6
total			100

2. PCR condition

95° 15 minutes

95° 1 minute , 45° 1 minute , 72° 2 minute 35 cycles

72° 7 minute

3.1.5 PIGR exon 5

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer	10X	1X	10
$MgCl_2$	25 mM	1.5 mM	6
Taq (Promega)	5 U/μl	2 U/100μl	0.4
Primer Ex-5Fy	10 μM	0.4 μM	4
Primer Ex-5Ry	10 μM	0.4 μM	4
DNA	50 ng/μl	100 ng	10

	Strock concentration	Working concentration	using(μl)
dH_2O			63.6
total			100

2. PCR condition

95° 5 minutes

95° 1 minute , 45° 1 minute , 72° 2 minute 35 cycles

72° 7 minute

3.1.6 PIGR exon 7

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer	10X	1X	10
$MgCl_2$	25 mM	1.5 mM	6
Taq (Promega)	5 U/μl	2 U/100μl	0.4
Primer Ex-7Fy	10 μM	0.5 μM	5
Primer Ex-7Ry	10 μM	0.5 μM	5
DNA	50 ng/μl	100 ng	10
dH_2O			61.6
total			100

2. PCR condition

95° 4 minutes

95° 1 minute , 55° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

3.1.7 PIGR exon 8

1.PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer	10X	1X	10
$MgCl_2$	50 mM	1.5 mM	3
Taq (Gibco)	5 U/μl	2 U/100μl	0.4
Primer seq Ex8-F	10 μM	0.4 μM	1
Primer pIgR Ex8R	10 μM	0.4 μM	1
DNA	50 ng/μl	200 ng	20
dH_2O			62.6
total			100

2. PCR condition

95° 5 minutes

95° 1 minute , 60° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

3.1.8 PIGR exon 9,10

1. PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2
10Xbuffer	10X	1X	10
$MgCl_2$	50 mM	1.5 mM	3
Taq (Gibco)	5 U/μl	2 U/100μl	0.4
Primer pIgR E 9 F	10 μM	0.8 μM	2
Primer pIgR-Ex10:B R	10 μM	0.8 μM	2
DNA	50 ng/μl	200 ng	20
dH_2O			60.6
total			100

2. PCR condition

95° 5 minutes

95° 1 minute , 50° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

3.1.9 PIGR exon 11

1. PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	2

	Strock concentration	Working concentration	using(μl)
10Xbuffer	10X	1X	10
$MgCl_2$	50 mM	1.5 mM	3
Taq (Gibco)	5 U/μl	2 U/100μl	0.4
Primer seq Ex 11-F	10 μM	0.4 μM	1
Primer seq Ex 11-R	10 μM	0.4 μM	1
DNA	50 ng/μl	200 ng	20
dH_2O			62.6
total			100

2. PCR condition

95° 5 minutes

95° 1 minute , 55° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

3.2 Purify PCR product by QIAquick gel extraction kit protocol.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel.
3. Incubate at 50°C for 10 min (or unit the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.

4. After the gel slice has dissolved completely, check that the color of mixture is yellow (similar to Buffer QG without dissolved agarose).

5. Add 1 gel volume of isopropanol to the sample and mix.

6. To blind DNA, pipet the sample onto the QIAquick column and apply vacuum. After the sample has passed though the column, switch off vacuum source.

7. Add 0.5 ml of Buffer QG to QIAquick column and apply vacuum.

8. To wash, add 0.75 ml of Buffer PE to QIAquick column and apply vacuum.

9. Transfer QIAquick column to a clean 1.5 ml microfuge tube or to a provided 2 ml collection tube. Centrifuge for 1 min at more than 10,000xg (13,000 rpm).

10. Place QIAquick column in a clean 1.5 ml microfuge tube.

11. To elute DNA, add 50 /µl of buffer EB or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min at more than 10,000xg. Alternatively, for increased DNA concentration, add 30 µl elution buffer, let stand for 1 min, and then centrifuge for 1 min.

3.3 Sequencing reaction were performed by Bio Service Unit (BSU) which using various primer.

Exon 1 were sequenced by Int-EX1F.

Exon 2 were sequenced by Int-EX2F.

Exon 3 were sequenced by Int-EX3R.

Exon 4 were sequenced by Int-EX4F1 and Int-EX4F2.

Exon 5 were sequenced by Int-EX5F1.

Exon 7 were sequenced by Int-EX7F.

Exon 8 were sequenced by pIgRE8 F and pIgRE8 R.

Exon 9 were sequenced by pIgR-E9:B R.

Exon 10 were sequenced by pIgR-E10:B F.

Exon 11 were sequenced by pIgR-E11:B F and pIgR-E11:B R.

3.4 Comparing the sequence by BLAST program and finding the mutation

4.SNPs finding

Using *PIGR* mRNA s62403 sequence searched from GenBank as a template, for SNP finding by strategies below.

1. Sequences which homology with *PIGR* mRNA were percieved from using BLAST program in http://www.ncbi.nlm.gov.
2. High frequent mismatch nucleotide more than 3 times derived from BLAST program can be defined as the SNPs of *PIGR* gene.
3. SNPs which have amino acid change, missense mutation, were selected to be candidate ones.

5.Multiplex ARMs for detection SNPs

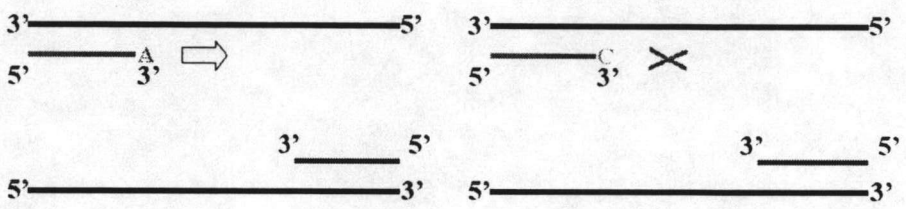
5.1 Primer design

1. ARMS primer should be oligonucleotides of around 30 bases and 50% G+C content.
2. Three prime of primer was a SNP position and added the deliberate mismatch at the penultimate base of the ARMS primer.
3. Deliberate mismatch at the penultimate were showed in appendix C.[92]

4. One SNP position had 3 primers. Obviously, we described by picture.

Example There are two possibility nucleotides in this SNP position, A and C.

Figure 7A



As shown in figure 7A, A nucleotide at 3' primer could be amplification. Hence, this SNP position had nucleotide A.

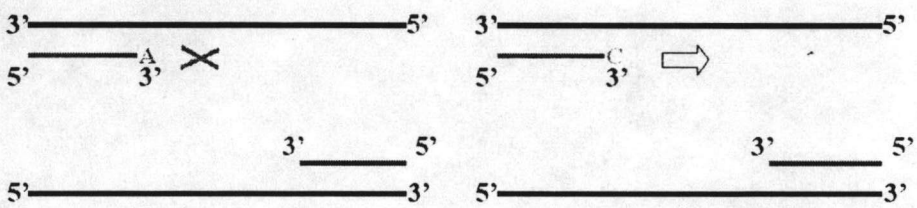


Figure 7B

As shown in figure 7B, C nucleotide at 3' primer could be amplification. Thus, this SNP position had nucleotide C. Consistently, this SNP was heterozygote if A and C at 3' primer can amplify the PCR product.

5.2 Multiplex ARMS PCR

Using triplex ARMS PCR to detect SNPS at 1093, 1739, and 2150.

5.2.1 PCR component of set A

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.5 mM	1

	Strock concentration	Working concentration	using(μl)
10Xbuffer	10X	1X	2
$MgCl_2$	25 mM	4 mM	3.2
Taq Gold (Perkin)	5 U/μl	1U	0.2
Primer 1249 F_A	20 μM	0.2 μM	0.2
Primer 1249 R	20 μM	0.2 μM	0.2
Primer 1895 F_A	20 μM	0.1 μM	0.1
Primer 1895 R	20 μM	0.1 μM	0.1
Primer 2306 R_1	20 μM	0.2 μM	0.2
Primer 2306 F	20 μM	0.2 μM	0.2
DNA	50 ng/μl	250 ng	5
dH_2O			7.6
total			20

5.2.2 PCR condition of set A

95° 10 minutes

95° 1 minute , 65° 1 minute , 72° 1 minute 30 cycles

72° 7 minute

5.2.3 PCR component of set B

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.5 mM	1

	Strock concentration	Working concentration	using(μl)
10Xbuffer	10X	1X	2
$MgCl_2$	25 mM	4 mM	3.2
Taq Gold (Perkin)	5 U/μl	1U	0.2
Primer 1249 F_B	20 μM	0.2 μM	0.2
Primer 1249 R	20 μM	0.2 μM	0.2
Primer 1895 F_B	20 μM	0.1 μM	0.1
Primer 1895 R	20 μM	0.1 μM	0.1
Primer 2306 R_2	20 μM	0.2 μM	0.2
Primer 2306 F	20 μM	0.2 μM	0.2
DNA	50 ng/μl	250 ng	5
dH_2O			7.6
total			20

5.2.4 PCR condition of set B

95° 10 minutes

95° 1 minute , 65° 1 minute , 72° 1 minute 30 cycles

72° 7 minute

Using simplex ARMS PCR to detect SNP at 966.

5.2.5 PCR component of set A at 966

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	0.4
10Xbuffer	10X	1X	2
$MgCl_2$	25 mM	1.5 mM	1.2
Taq Gold (Perkin)	5 U/μl	2 U/100μl	0.08
Primer 1122 R_1	10 μM	0.3 μM	0.6
Primer 1122 F	10 μM	0.3 μM	0.6
DNA	50 ng/μl	200 ng	1
dH_2O			13.72
total			20

5.2.6 PCR conditiont of set A at 966

95° 10 minutes

95° 1 minute , 65° 1 minute , 72° 1 minute 30 cycles

72° 7 minute

5.2.7 PCR component of set B at 966

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	0.4
10Xbuffer	10X	1X	2
$MgCl_2$	25 mM	1.5 mM	1.2

	Strock concentration	Working concentration	using(μl)
Taq Gold (Perkin)	5 U/μl	2 U/100μl	0.08
Primer 1122 R_2	10 μM	0.3 μM	0.6
Primer 1122 F	10 μM	0.3 μM	0.6
DNA	50 ng/μl	200 ng	1
dH_2O			13.72
total			20

5.2.8 PCR conditiont of set b at 966

95° 10 minutes

95° 1 minute , 65° 1 minute , 72° 1 minute 30 cycles

72° 7 minute

Same sample was amplify 2 tubes;

- Tube 1 amplification with primer set A.

- Tube 2 amplification with primer set B.

5.2.9 Run 3% agarose gel electrophoresis,data collection and analysis.

6.DNA sequencing and finding new SNP.

As a result of ARMS, some sample could not amplify with both primer sets and PCR product not stable. We designed to sequence for explain this problem. Likewise, preparation and purify samples for sequencing were used the method in 3.2 items.

7.PCR and RFLP

From multiplex ARMS PCR, 966 and 2150 did not the polymorphism in this sample population and 1739 PCR product not stable. Besides from the result of 6 items, I would find new SNP at 1773. Finally, I designed PCR and RFLP to detect SNPs at 1739 and 1773.

7.1 PCR component. Both SNPs had PCR component as below.

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	0.4
10Xbuffer	10X	1X	2
$MgCl_2$	25 mM	1.5 mM	1.2
Taq (Perkin)	5 U/μl	2 U/100μl	0.08
Primer E7 FORSNP-F	20 μM	0.2 μM	0.2
Primer R1895	20 μM	0.2 μM	0.2
DNA	50 ng/μl	200 ng	1
dH_2O			13.92
total			20

7.2 PCR condition

95° 5 minutes

95° 1 minute , 60° 1 minute , 72° 1 minute 35 cycles

72° 7 minute

7.3 RFLP reaction by *HgaI* enzyme for SNP1739 detection.

PCR product	8 μl
10XNE buffer1	0.5 μl
Enzyme *HgaI*	0.2 μl
dH_2O	1.3 μl
Total	10 μl

-Incubate 37°C overnight.

7.4 RFLP reaction by *MspI* enzyme for SNP1773 detection.

PCR product	8 μl
10XNE buffer2	0.5 μl
Enzyme *MspI*	0.2 μl
dH_2O	1.3 μl
Total	10 μl

-Incubate 37°C overnight.

7.5 SNP1739 detection was run in 2% agarose gel electrophoresis.

7.6 SNP1773 detection was run in 12% nondenaturing polyacrylamide gel electrophoresis.

7.7 Data collection and analysis.

8. Three prime end of UTR polymorphism.

Fabregat I and colleagues reported 3' UTR of rat Pigr gene having function pleiotropy.[93] Thus, I designed to detect length polymorphism at 3'UTR of human *PIGR* gene by PCR and polyacrylamide gel electrophoresis.

8.1 Using 20 DNA samples.

8.2 End-labeled of forward primer (PIGR UTR F)

10X T4 buffer	1 μl
Enzyme T4 kinase	1 μl
Primer PIGR UTR F(20 μM)	5 μl
dH_2O	2 μl
[γ^{32}P] ATP (3000Cli/mmol)	1 μl
Total	10 μl

-Incubate 37°C 1-2 hours.

8.3 PCR component

	Strock concentration	Working concentration	using(μl)
dNTP	10 mM	0.2 mM	0.2
10Xbuffer	10X	1X	1
$MgCl_2$	25 mM	1.5 mM	0.6
Taq (Perkin)	5 U/μl	2 U/100μl	0.04
Labeled-PIGR UTR F	10 μM	0.1 μM	0.2
Primer PIGR UTR R	20 μM	0.1 μM	0.1
DNA	50 ng/μl	200 ng	2
dH_2O			5.89
total			10

8.4 PCR condition

95° 5 minutes

95° 1 minute , 60° 1 minute , 72° 1 minute 25 cycles

72° 7 minute

- Two microliters of each PCR product was mixed with 1 μl formamide-loading dye, heated at 95°C for 2 minutes, put on ice for 30 seconds, and loaded onto 6% denaturing polyacrylamide gel.
- After electrophoresis the wet gel was transferred to filter paper, wrapped with saran wrap and exposed to a phosphorus screen.
- The bands were visualized by using Image QuaNT solfware.

9. Statistic analysis.

The relative risk was estimated by the odds ratio (OR) method, in order to determine the relationship between genotype of *PIGR* gene and NPC development. Furthermore, 95% confidence interval (CI) was calculated to determine the statistical significance of data. Exact method from Epit info version6 program was used to calculate OR and 95% CI and EH program was used to estimate haplotype frequencies in the population. The number of cases with haplotype GC/AT or GT/AC from cases with heterozygote from both 1093G→A and 1739T→C were estimated according to the probability of each combination. The calculation was performed according to these formulas GC/AT=NX[EH(GC)XEH(AT)]/{[EH(GC)XEH(AT)]+[EH(GT)XEH(AC)]}, and GT/AC=N[EH(GT)XEH(AC)]/{[EH(GC)XEH(AT)]+[EH(GT)XEH(AC)]}. N is number of cases with compound heterozygote, EH(haplotype) is haplotype frequency from EH calculation of each indicated haplotype. GC, AT, AC and GT are haplotype alleles of 1093G→A and 1739T→C. The difference of haplotypes between groups was estimated by $T(x^2/2)$ = ln(L,group1)+ln(L,group2)-ln(L,group1+group2). [94]