

# Molecular genetic detection of susceptibility to malignant hyperthermia in Belgian families

L. HEYTENS

**Abstract :** Malignant hyperthermia is an autosomal dominant myopathy triggered by volatile anesthetics or succinylcholine in susceptible persons. While in vitro contracture testing (IVCT) is the gold standard to establish malignant hyperthermia (MH) susceptibility, genetic analysis is increasingly used to diagnose this condition. This work aimed to determine the frequency and distribution of ryanodine receptor (RYR1) mutations in the Belgian MH-population as investigated by IVCT in our centre, as well as the discordance rates between the 2 techniques.

Sequence analysis of 16 RYR1-exons in 29 selected families resulted in the detection of 10 mutations (4 Gly341Arg, 2 Arg614Leu, and 1 Cys35Arg, Arg614Cys, Arg2163Cys and Arg2435His). Discordance between IVCT and mutation analysis was observed in only 6 out of 96 individuals from 4 different families. No mutation-positive/ IVCT-negative diagnosis was found. Genetic evaluation of RYR1-mutations can secure a diagnosis and aid in genetic counselling of individual family members but only in those families in which significant clinical information is present, as well as phenotyping by IVCT has been realized.

**Key words :** Malignant hyperthermia ; ryanodine receptor ; in vitro contracture test ; genetic diagnosis.

## INTRODUCTION

Malignant Hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle that manifests as acute rhabdomyolysis during general anesthesia when susceptible individuals are exposed to volatile anesthetics and/or succinylcholine.

This potentially life-threatening event reflects disturbed skeletal muscle calcium homeostasis as a result of defects in the genes coding for the proteins involved in skeletal muscle excitation-contraction coupling, e.g. the ryanodine receptor (RYR) and – rarely – the dihydropyridinereceptor (DHPR).

Susceptibility to MH is normally diagnosed by in vitro contracture testing (IVCT) with caffeine and halothane. This test has been standardised across Europe by the European Malignant

Hyperthermia Group (EMHG) and shows a high degree of sensitivity (99%) and specificity (93.5%) (6). However, the invasive nature of the muscle biopsy hampers very much its widespread use as a screening instrument. Therefore it has been a long-term goal to assess whether DNA based diagnosis for MH is feasible.

Over the last 14 years the Malignant Hyperthermia laboratory of the University of Antwerp - the national reference centre for MH-diagnosis and member of the European Group for Malignant Hyperthermia - has performed over 400 in vitro contracture tests to establish the MH-phenotype in referred probands and their family members allowing us to establish a phenotype databank concerning a large number of families with at least two generations investigated.

The specific objectives of this study were :

1. to obtain a comprehensive assessment of the prevalence of 22 – at that time internationally published – ‘causative’ mutations in the Belgian population
2. to compare these genetic data with the previously obtained in vitro contracture test results and study the genotype/phenotype correlation
3. finally, to study the feasibility to offer genetic counselling to the families in which a causative RYR1 mutation was found.

## PATIENTS AND METHODS

### Phase I

During the initial phase of the study we assessed the overall frequency of the 15 RYR1

L. HEYTENS, Verantwoordelijke Onderzoeksgroep Maligne Hyperthermie, University of Antwerp Campus Drie Eiken, Wilrijk, Belgium.

**Correspondence address :** L. Heytens, University of Antwerp Campus Drie Eiken, Gebouw T lokaal 0.04, Universiteitsplein 1, B-2610 Wilrijk, Belgium.

mutations published in the original guidelines of the EMHG-group (10). For this mutation screening of RYR1, 29 Belgian families were selected on the basis of the information available in our clinical and in vitro contracture test (IVCT)-database. All families had been referred to our MH laboratory after a suspected clinical MH episode in a relative. Susceptibility to MH was diagnosed by IVCT with caffeine and halothane according to the protocol of the European Malignant Hyperthermia Group (EMHG). The IVCT requires a sample of skeletal muscle tissue, which is exposed in vitro to incremental doses of the testing agents caffeine and halothane, and the contracture response is measured. The individual patients were classified according to three IVCT diagnoses : MH susceptible (MHS), MH normal (MHN) or MH equivocal (MHE). The test is considered positive (MHS) if a sustained contracture of at least 2 mN is obtained in two different muscle bundles at caffeine concentrations of 2 mM or less, and halothane concentrations of 2 Vol% or less. Normal individuals (MHN) do not react at the threshold concentrations of either agent. The result is called equivocal (MHE) when a significant contracture (2 mN or more) is obtained with only one test substance e.g. MHEh if reacting to halothane and MHEc when reacting to caffeine only. Patients classified as MHS and MHE are considered clinically at risk to MH.

At the time of the muscle biopsy blood was sampled from all individuals and DNA extracted and stored. Informed consent regarding this blood sampling and the ensuing DNA analysis was obtained from all patients. The total number of DNA-samples analyzed was 96 and ranged from 2-13 in the individual families.

Intron based *RYR1* PCR primers were designed for the 11 exons described in ROBINSON *et al.* (7) using SNPbox. This is a modular software package that automates the design of PCR primers for large-scale amplification and sequencing projects in a standardized manner resulting in high-quality PCR amplicons with a low failure rate (12).

In view of the high cost, sequence analysis was performed in 24 index individuals only. In families 4,5,9,19 and 23 with a previously detected mutation by other techniques a new analysis was not performed at this stage.

Standard PCR reactions were performed and amplification products were purified with Exo-SapIT (Amersham Biosciences). The DNA sequence of both strands was determined by cycle

sequencing using the 'BigDye Terminator Cycle Sequencing' kit v3.1 and analyzed on an ABI3730 DNA analyzer (Applied Biosystems). Sequences were using novoSNP (11).

During this first part of our study however, the 'Guidelines for the molecular detection of susceptibility to malignant hyperthermia' as published in the Br J Anaesth in 2001 (10) were updated and 22 mutations in 16 exons were considered to be causative of MH ([www.EMHG.org](http://www.EMHG.org)). Therefore the 5 extra exons containing the 'new' mutations were also sequenced applying the same technique.

Table 1

Listing of the 29 selected families indicating the mutations found in the respective index cases with both SNPbox (families 7,8,11, 20 and 29) and previously known results (families 4,5,9,19 and 23). MHN refers to the particular family number in our database ; MHN.x refers to the particular individual investigated, most often the proband

Family number + individual	Mutation detected
MH1.10	-
MH3.6	-
MH4	Gly341Arg
MH5	Gly341Arg
MH6.3	-
MH7.3	Arg614Leu
MH8.1	Arg614Leu
MH9	Gly341Arg
MH10.6	-
MH11.3	Arg2435His
MH12.3	-
MH13.1	-
MH14.3	-
MH15.6	-
MH16.3	-
MH17.1	-
MH18.1	-
MH19	Val2168Met
MH20.1	Cys35Arg
MH22.3	-
MH23	Gly341Arg
MH24.4	-
MH25.2	-
MH26.1	-
MH27.1	-
MH28.1	-
MH29.1	Arg614Cys
MH32.1	-
MH33.1	-

RESULTS

With the sequencing technique used and covering 16 exons, mutations were found in 5 out of 24 individuals.

Phase II

In those families in which one of these 22 mutations was identified in the key individual, all other family members were screened for that particular mutation.

To perform the mutation analysis in these families the pyrosequencing technology was used. This technology is a non-electrophoretic DNA-sequencing method using “sequencing by synthesis” and is suitable for the rapid detection of simple nucleotide polymorphisms (SNP’s). Short runs of sequence around each polymorphism are generated, allowing for internal control of each sample (12).

Phase III

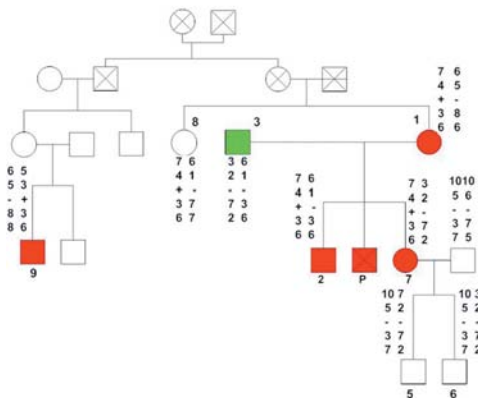
A main concern for MH-diagnosis – either by IVCT or DNA analysis – is the reported

discordance between IVCT phenotype and RYR1 genotype. In some families individuals classified as MH-susceptible by the IVCT have been found not to carry the familial RYR1-mutation. More rarely – but with more potentially dangerous consequences – some cases considered MH normal by the IVCT have been reported to carry a high risk haplotype and/or mutation. The discordance rate was reported to be 8% in a large European study with 196 families (6).

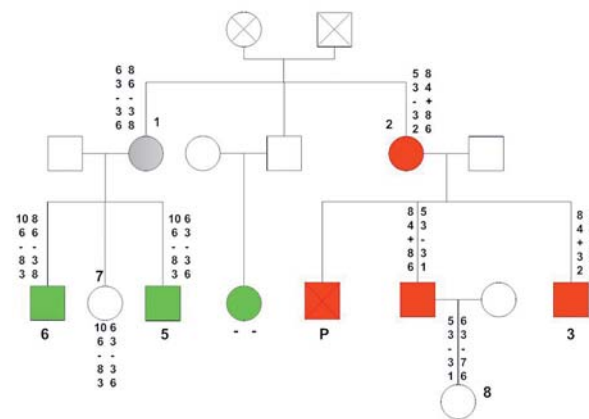
We therefore assessed the degree of discordance in our population by analyzing the RYR1 genotype/IVCT result in all 96 individuals.

Discordance was observed in 6 out of 96 individuals (6,3%) from 4 different families. No mutation-positive/negative IVCT diagnosis was found. All individuals either had an MHE/mutation-negative diagnosis (4 individuals : fam 5.indiv9, fam 7.indiv1, fam 9.indiv9, fam 19.indiv12) or a MHS /mutation negative diagnosis (2 individuals : fam 8.indiv4, fam 11.indiv6). Upon re-examining the IVCT data of these six discordant individuals, 4 test results showed borderline contractures of 1-2 mN ; one IVCT was characterized by poor to borderline viability of the different muscle specimen and borderline contractures

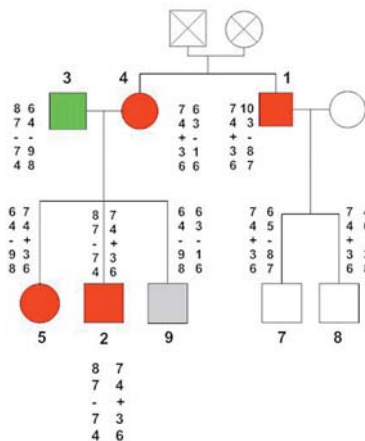
Family 4 Gly341Arg mutation



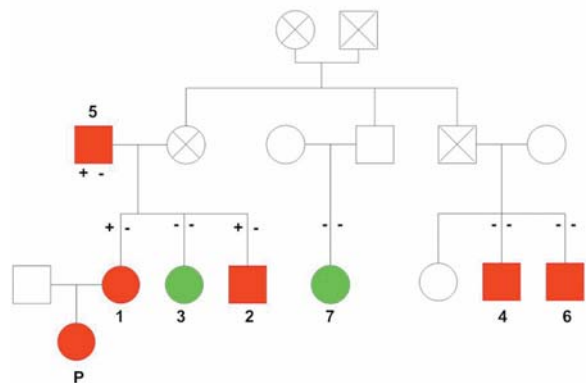
Fam 7 Arg614Leu mutation



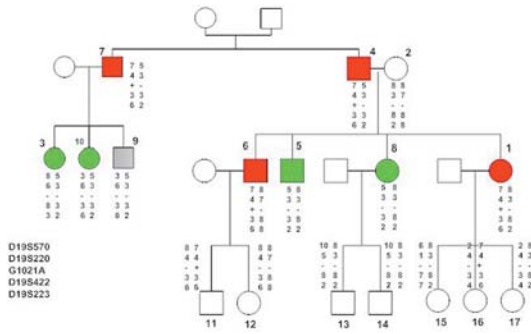
Family 5 Gly341 Arg mutation



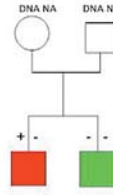
Family 8 Arg614Leu mutation



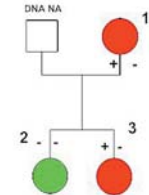
Family 9 Gly341Arg mutation



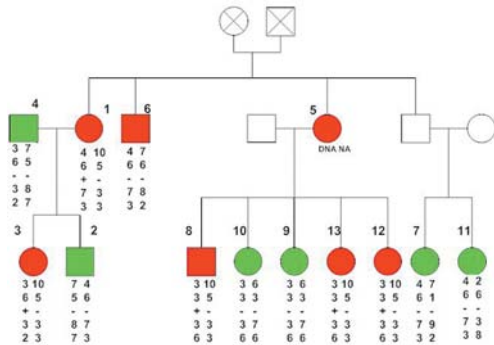
Family 20 Cys35Arg mutation



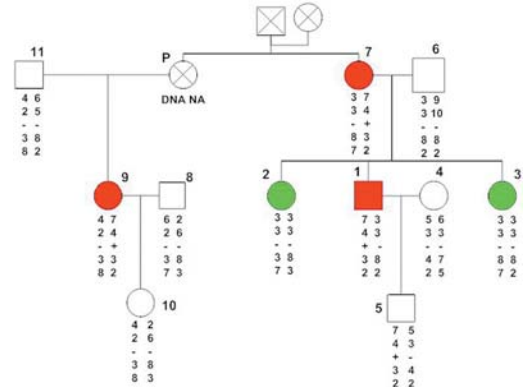
Family 29 Arg614Cys mutation



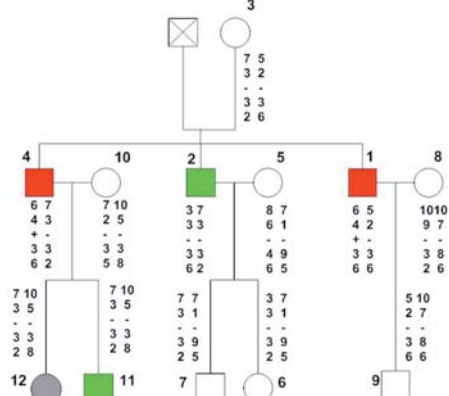
Family 11 Arg2435His mutation



Family 23 Gly 341Arg mutation



Family 19 Val2168Met mutation



Figs. 1-9. — Pedigrees of the 10 families in which a mutation was found (SmartDraw version 2007). The particular mutation found in each family is indicated in the upper left hand corner. The presence of the mutation in an individual is indicated by (+ -), its absence by (- -), either alone or included in the haplotypes obtained within that family (numerical series). The phenotype of the individual family members as tested by in vitro contracture testing is given by symbols and color codes. Malignant Hyperthermia Susceptible individuals are represented by red squares (male) and circles (female); Malignant Hyperthermia Non-Susceptible individuals by green squares and circles. White squares and circles represent individuals not tested by IVCT; gray squares and circles represent individuals with an equivocal IVCT. Crossed squares and circles indicate deceased individuals. The numbers given above the individual symbols correspond to the patients' number in our database; a "P" indicates the proband e.g. the person having presented with a clinical MH-episode.

(fam 8.indiv4) ; but one IVCT was clearly positive for both halothane (4 mN at 2 Vol% Hal) and caffeine (2 mN at 2 mM caffeine) (fam 11.indiv6).

Phase II and III results

DISCUSSION

Malignant Hyperthermia during anesthesia reflects abnormal calcium homeostasis as a result of missense mutations in the ryanodine receptor gene

(RYR1) and – rarely – the dihydropyridinereceptor gene (DHPR). The RYR1 gene encodes a protein of 5.038 amino acids and comprises 106 exons. It is one of the largest genes known.

Genetically, MH-susceptibility exhibits an autosomal dominant mode of inheritance with an estimated prevalence of 1 in 8,500. It has been shown to demonstrate both locus and allelic heterogeneity with 5 distinct susceptibility loci so far identified.

Fortunately, the majority of families demonstrate linkage to one locus on chromosome

19q13.1, known since quite some time to correspond to the ryanodine receptor gene (RYR1). The remaining loci have been identified only in isolated families.

To date over 100 RYR1 mutations have been reported to be linked to MH-susceptibility, only part of which until now have been convincingly shown to be 'causative of MH'.

Mutations in the RYR1 gene have been found in association with three different congenital myopathies (central core diseases (CCD), multiminicore disease (MmD) and a single case of congenital myopathy characterized on histology with rods and cores.

Previous to this study, 11 Belgian families were investigated for the presence of a limited number of mutations in RYR1 (Arg146Cys, Gly248Arg, Arg2434His, Arg163Cys, Ile403Met and Gly341Arg). The detection techniques used for this screening were those available at that time and included allele-specific PCR, SSCP and RFLP analysis. This preliminary screening showed the Gly341Arg mutation to be present in 4 out of 11 families; in a fifth family the Arg2163His mutation was found. After re-analysis of the older results, it became clear that this latter mutation had to be reclassified as a Val2168Met (6502G/A) mutation.

Taking into account the 5 new families during this sequencing analysis, we have detected one of the 22 'causative' RYR1 mutations in 10 out of the 29 families. The most prevalent mutation in our patients studied is the Gly341Arg mutation (5 families) followed by the Arg614Leu mutation (2 families).

To be considered truly 'causative', these mutations should fulfil specific criteria at the genetic level as well as regarding its functional expression ([www.EMHG.org](http://www.EMHG.org)). At the time of this study, 22 RYR1 mutations fulfilled these criteria. These mutations appear to cluster in 3 regions namely the N-terminal region bp 1-700 and a second central region bp 2300-2500. Both correspond to gene subunits coding for the cytoplasmic foot structure of RYR. A third region of the gene, the C-terminal region has also been found to harbour mutations found in MH as well as in central core disease, a structural myopathy that strongly predisposes to MH-susceptibility. This third region is believed to code for the transmembrane pore.

In 2005/2006, 6 more RYR1 mutations have been added to the EMHG-list of causative mutations (Arg163Leu, Thr2206Arg, Gly2375Ala,

Ala2428Thr, Arg2454Cys and Arg2454His) and it can certainly be anticipated that even more will be included in the near future. This ever evolving heterogeneity represents one of the major shortcomings of the current molecular genetic techniques used in the detection of MH-susceptibility and explains why the present capacity of DNA testing for MH is unclear with huge differences in overall mutation detection rates between varying between 30 (7) and 86 % (1). The overall detection rate is certainly influenced by the technique used and the extent to which the RYR1 gene is sequenced. The most recent surveys report detection rates as high as 70 (8) and 86% (1). In this last study, all 106 exons were sequenced compared to the 16 in our study. It is however unclear to what extent these different sequence variants are truly causative of MH.

A European survey including data from 10 MH units published in 2003 showed that the most frequent RYR1 mutations in Europe are Gly341Arg, Arg614Cys and Gly2434Arg accounting respectively for 14.8, 28.6 and 34.2% of the mutation positive cases (7). In the United States, and Japan, the distribution as well as the frequency of the individual mutations was reported to be markedly different (9, 3). This extremely high RYR1 allelic heterogeneity has also been found between European labs (7). For this reason the question whether 'MH screening kits' will ever be sufficiently sensitive in isolation of the phenotypic data obtained by IVCT is unanswerable at the moment.

Phenotype/genotype discordance was observed in 6 out of 96 individuals from 4 different families (6%). No mutation-positive/IVCT negative combination was found in this study indicating that a false negative IVCT diagnosis is until now not present in our series thereby confidently minimising the possibility of a potentially fatal false negative diagnosis by IVCT.

Conversely the 4 discordant individuals with borderline contractures probably indicate a false positive IVCT MH-diagnosis indicating that the European criteria used are biased toward positive diagnoses for reasons of high sensitivity, with an accepted loss of specificity.

When reviewing the original older charts and following the exclusion of potentially erroneous data because of technical reasons however, 2 tests still would meet the current EMHG-criteria and thus indicate MH susceptibility by in vitro contracture testing standards.

Even though mutations in the RYR1 gene are associated with the majority of reported MH cases,

current knowledge about the allelic heterogeneity of this myopathy emphasises that DNA screening for MH is still not feasible in isolation of the phenotypic data obtained by history and IVCT. Only when significant clinical information as well as phenotyping by IVCT has been realized in a family can genetic evaluation of RYR1 mutations secure a diagnosis and aid in genetic counselling of individual family members. The low discordance rate in the 10 families with a mutation is a good indication that at least in these families further genotyping is useful and can avoid the use of a muscle biopsy.

Finally, over a decade of intensive research into the functional consequences of how these missense mutations affect the functional properties of sarcoplasmic reticulum Ca-release have significantly advanced our understanding of calcium homeostasis in muscle and how dysfunction of this system can lead to neuromuscular disorders such as MH, CCD and MmD.

A recent review provides an excellent synopsis of the preoperative anesthetic work-up, the potential adverse effects of anesthetics on diseased skeletal muscle as well as suggested anaesthetic procedures in patients with various neuromuscular diseases (4).

#### Acknowledgments

The mutation analysis was performed by the "Applied Molecular Genomics Group", Department of Molecular Genetics VIB8 (Scientific Director Prof C Van Broeckhoven ; Supervision of analysis Prof Dr Ir J Del-Favero) University of Antwerp – campus CDE, Building V, Room 1.14 Universiteitsplein 1, B 2610 Wilrijk. Website : [www.molgen.ua.ac.be](http://www.molgen.ua.ac.be).

We gratefully acknowledge the financial support from the research grant 2003 of the Society for Anesthesia and Reanimation of Belgium.

#### References

1. Galli L., Orrico A., Lorenzini S., Censini S., Falciani M., Covacci A., Tegazzin V., Sorentino V., *Frequency and localization of mutations in the 106 exons of the RYR1 gene in 50 individuals with malignant hyperthermia*, HUM. MUTAT., **27** (8), 830-834, 2006.
2. Girard T., Urwyler A., Censier K., Mueller C. R., Zorzato F., Treves S., *Genotype-phenotype comparison of the Swiss malignant hyperthermia population*, HUM. MUTAT., **18** (4) : 357-358, 2001.
3. Ibarra M. C. A., Wu S., Murayama K., Minami N., Ichihara Y., Kikuchi H., Noguchi S., Hayashi Y. K., Ochiai R., Nishino I., *Malignant hyperthermia in Japan : mutation screening of the entire ryanodine receptor type 1 gene coding region by direct sequencing*, ANESTHESIOLOGY, **104** (6), 1146-1154, 2006.
4. Klingler W., Lehmann-Horn F., Jurkat-Rott K., *Complications of anesthesia in neuromuscular disorders*, NEUROMUSC. DISORDERS, **15**, 195-206, 2005.
5. Monnier N., Kozak-Ribbens G., Krivosic-horber R., Nivoche Y., Qi D., Kraev N., Loke J., Sharma P., Tegazzin V., Figarella-Branger D., *et al.*, *Correlations between genotype and pharmacological, histological, functional, and clinical phenotypes in malignant hyperthermia susceptibility*, HUM. MUTAT., **26** (5) : 413-425, 2005.
6. Ording H., *In vitro contracture test for diagnosis of malignant hyperthermia following the protocol of the European MH Group : results of testing patients surviving fulminant MH and unrelated low-risk subjects*, ACTA ANAESTHESIOL. SCAND., **41**, 955-966, 1997.
7. Robinson R. L., Anetseder M. J., Brancadoro V., Van Broeckhoven C., Carsane A., Censier K., Fortunato G., Girard T., Heytens L., Hopkins P. M., *et al.*, *Recent advances in the diagnosis of malignant hyperthermia susceptibility : how confident can we be of genetic testing ?*, EUR. J. HUM. GENET., **11**, 342-348, 2003.
8. Sambuughin N., Holley N., Muldoon S., Brandom B. W., de Bantel A. M., Tobin J. R., Nelson T. E., Goldfarb L. G., *Screening of the entire ryanodine receptor type 1 coding region for sequence variants associated with malignant hyperthermia susceptibility in the north American population*, ANESTHESIOLOGY, **102**, 515-521, 2005.
9. Sambuughin N., Sei Y., Gallagher K. L., Wyre H. W., Madsen D., Nelson T. E., Fletcher J. R., Rosenberg H., Muldoon S. M., *North American malignant hyperthermia population : screening of the ryanodine receptor gene and identification of novel mutations*, ANESTHESIOLOGY, **95** (3), 594-599, 2001.
10. Urwyler A., Deufel T., McCarthy T., West S., *Guidelines for the molecular detection of susceptibility to malignant hyperthermia*, BR. J. ANAESTH., **86**, 283-287, 2001.
11. Weckx S., Del-Favero J., Rademakers R., Claes L., Cruts M., De jonghe P., Van Broeckhoven C., De Rijk P., *Novo SNP, a novel computational tool for sequence variation discovery*, GENOME RES., **15** (3), 436-442, 2005.
12. Weckx S., De Rijk P., Van Broeckhoven C., Del-Favero J., *SNPbox : web-based high-throughput primer design from gene to genome*, NUCLEIC ACIDS RES., **32** (Web Server issue) : W170-172, 2004.