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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 91/107G

ICGEB ref. #: CRP/BUL90-06

Project initiation: 1991

Project termination: 1994

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TERMINAL EVALUATION REPORT

Part I

Title of Project Cystic Fibrosis in Bulgaria

Keywords: Cystic Fibrosis, CFTR gene, Mutations, Genetic Prevention

UNIDO contract # 91/107G **ICGEB ref. #:** CRP/BUL90-06

Project initiation: 1991 **Project termination:** 1994

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Abstract:

One hundred fourty five families with cystic fibrosis were investigated in the course of this project which aimed to identify the mutations causing the disease in the heterogeneous population of Bulgaria, to evaluate the phenotypic effects of rare mutations, investigate the population genetics of cystic fibrosis within Bulgaria and contribute the worl-wide epidemiological studies and outline the strategy for future prevention in the country. The disease-causing mutation has been identified in 86% of CF alleles and a pettern of extensive allelic heterogeneity has emerged. Twelve CF mutations have been identified for the first time in this study, some of which have been found subsequently in patients in neighbouring countries. A finding of particular interest is the identification of double-mutant CF alleles which carry two independent disease-causing mutations and which may turn out to be much more common than generally assumed. Genotype-phenotype correlations in Bulgarian patients with rare CF mutations have been investigated in the framework of several international studies. A strategy for secondary prevention has been worked out based on the findings of this project and its implementation in the study of newly referred CF families is already underway. A pilot screening for the delection of delF508 carriers has revealed highly significant inter-ethnic differences in Bulgaria with serious implications for genetic counselling and for the targeting of primary prevention of cystic fibrosis in the country. The laboratory has participated in (and in some cases initiated) several studies aiming to trace the origins and spread of CF mutations. Thanks to the project, technical developments such as the routine and highly reliable use of SSCP and sequencing analysis have taken place in the laboratory. A very important aspect of the project is the networking of the laboratory with the world-wide, the European and several local consortia involved in the study of cystic fibrosis. This has resulted in the allocation of EU funding for the Bulgarian participation in the European project on cystic fibrosis which will allow the extension of research and diagnostic activities in this field over the next three years. Over twenty publications have been produced as a result of this project and have contributed greatly to the track record of the laboratory, thus facilitating the submission of new grant proposals to funding agencies in the future.

Part 2

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

Cystic fibrosis (CF) is a common genetic disorder which results in chronic disability in childhood and early death in affected individuals. Its frequency in Bulgaria has been estimated at about 1 in 3 000. Preliminary investigations into the molecular basis of the disease carried out prior to the submission of the project had resulted in detection of the disease-causing mutation in less than 70% of CF chromosomes, thus limiting the potential for primary and secondary genetic prevention of the disease. In the meantime nearly 500 different mutations in the CFTR gene have been identified by the International Cystic Fibrosis Genetic Analysis Consortium. It has been demonstrated in a number of studies that the molecular basis of the disease is particularly heterogeneous in southern European populations where the main CF mutation (delF508) accounts for about 50% of all CF alleles and a large variety of rare mutations occur in the remaining disease alleles. An investigation into the molecular genetics of cystic fibrosis in southern European countries is therefore an essential element in designing the strategy of genetic prevention and also in the world-wide studies of the pathogenesis of this common disorder.

OBJECTIVES

* The main objective stated in the proposal was to identify the CF mutations which lead to the disease in the Bulgarian population whose genetic pool is representative of southern European as well as Slavic populations.

Other objectives:

* Evaluation of the effect of newly identified and rare mutations in the CFTR gene on disease phenotype, by relating molecular to clinical data. This type of information is important for a better understanding of the function of the CFTR protein and the pathogenesis of cystic fibrosis. Phenotype/genotype correlations also provide information on the value of molecular genetic analysis in predicting the clinical phenotype and severity of the disease in affected individuals.
* Obtaining information on the population genetics of cystic fibrosis by comparing mutation frequencies and associated haplotypes among different ethnic groups within the Bulgarian population and also by comparing the overall data on Bulgaria to those observed in other populations. This information is important for the detection of inter-ethnic variation inside Bulgaria and hence for the identification of target groups for genetic prevention. It is also important for the study of the epidemiology of cystic fibrosis worldwide and for investigating the origin and spread of CF mutations.
* Designing a strategy of CF prevention based on the findings of the present study. If a limited number of molecular defects are identified, a population-based carrier testing programme can be planned; if a large number of different mutations account for the disease in Bulgaria, secondary prevention in known CF families will be set as a target and will be carried out mainly through polymorphic analysis and possibly SSCP analysis.

METHODOLOGY

* Detection of known, common mutations in the CFTR gene by PCR amplification followed by dot blot hybridization, heteroduplex or direct restriction analysis, as appropriate.
* Single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) for the identification of fragments of the CFTR gene with aberrant electrophoretic mobility and a suspected underlying mutation in the region.
* Direct sequencing of regions found to be abnormal by SSCP or DGGE analysis and identification of sequence variation.
* RFLP and microsatellite analysis by PCR amplification and electrophoretic separation for the assignment of polymorphic alleles.

RESULTS
(compare against the set objectives)

RESULTS:

A total of 145 families with cystic fibrosis have been referred to the laboratory by pediatric departments from different regions of the country. Thus the total number of CF alleles investigated is 290.

Development of the appropriate methodology

* Dot-blot hybridization, direct restriction analysis and heteroduplex analysis were initially used for the identification of common CF mutations. In the course of the project, the former two techniques were found to be unsatisfactory and were therefore discontinued. On the other hand, heteroduplex analysis has been adapted for use with the Pharmacia PhastSystem where the analysis is performed on minigels with silver staining and is completed within less than one hour.
* Screrening for new mutations: SSCP versus DGGE analysis. Both techniques were tested and SSCP was selected as the method of choice because of its simplicity and feasibility. Detailed investigations aimed at improving the sensitivity of the technique were performed. These included a modification of experimental conditions and the use of higher percentage acrylamide for better resolution; the use of computer modelling of the conformation changes of mutant vs normal single strands for predicting the sensitivity of detection of SSCP analysis; comparison of the sensitivity of SSCP analysis with that of DGGE; direct assessment of the sensitivity of SSCP analysis in a collaborative experiment where the Laboratory of Molecular Pathology has received from other European laboratories anonymous samples from CF patients for "blind" SSCP analysis and identification of the mutant exons. As a result of these investigations, SSCP analysis is now a highly reliable technique in our hands (publications #6,9,16) and is used for both screening for unknown mutations and also for the identification of common mutations, thus replacing dot-blot hybridization and direct restriction analysis.
* Skills in the direct sequencing of PCR products were developed in the course of this project. The technique is now in routine use in the laboratory.
* In addition to the techniques envisaged in the original grant proposal, methods for the investigation of mRNA are being developed: extraction of total RNA from nasal epithelium and from blood cells, reverse transcription, PCR amplification and direct sequencing. This approach is used for the study of presumed splicing mutations, for investigating alleles of patients with a striking discrepancy between the nature of the mutation and the observed phenotype and for short-cutting the analysis of mutant alleles where the molecular defects remain unknown and where mutations deep in the intronic sequences might be present.

A systematic approach to mutation detection in newly referred CF patients and families elaborated in the course of the project

* Screening for known common mutations in the CFTR gene is performed through heteroduplex analysis of exon 10 (detection of delF508 and 1677delTA).
* This is followed by SSCP analysis of the mutation hot spots, namely exons 11, 7, 21, 17b and 31a. Known mutations are recognized through their characteristic electrophoretic patterns. At the same time, possible regions of new and/or rare mutations are identified by an abnormal SSCP pattern.
* Samples with suspected mutations in a specific exon are subjected to direct sequencing.

OBJECTIVE: Idnetification of mutations in the CFTR gene which cause cystic fibrosis in Bulgaria

* The mutation has been identified in 250 out of 290 CF chromosomes investigated. The data are shown in table 1. In 40 alleles (13.8%) the molecular defect remains unknown.
* Three mutations, namely delF508, N1303K and G542X account for nearly 70% of CF alleles in Bulgaria.
* In addition to the three common mutations, 24 different molecular defects have been identified in Bulgaria. The majority are very rare and have been detected in one or two CF alleles.
* Twelve new CF mutations have been described for the first time in this study (publications #14,15,20).

RESULTS
(compare against the set objectives)

OBJECTIVE: Study of genotype/phenotype correlations in patients with rare CF mutations

* Due to the rarity of the mutations, the evaluation of genotype/phenotype correlations in such patients requires international collaboration and the pooling of data collected in many centres. In the course of this project, the Laboratory of Molecualr Pathology in Sofia has participated in two collaborative studies initiated by other centres, namely a study of patients with the mutation N1303K and a study of patients with several rare mutations (publications #5,8). In addition, the Laboratory has organized three such studies on the following mutations: 1677delTA, R347P and G1069R (publications # 11,19,21).
* A special subgroup in the genotype/phenotype correlation study included patients with meconium ileus (MI), a severe complication of cystic fibrosis which occurs in about 15% of patients. The distribution of mutations was studied in 18 patients with MI (publication #18).
* The relationship between mutations in the CFTR gene and the clinical severity of cystic fibrosis is not simple and straightforward. Striking discrepancies are observed in some patients and are usually attributed to ill-defined "modifying factors". In the course of the project, four patients with three CF mutations were identified, i.e. some CF alleles are in fact double mutants and the presence of the additional mutation (which may occur more frequently than expected) may in fact be the modifying factor. All triple mutants detected in the present study carried newly identified, rare mutations in the double mutant chromosome (publication #19).

OBJECTIVE: Study of the population genetics of cystic fibrosis in Bulgaria

Overall frequency of CF carriers in Bulgaria

* Data on the overall frequency of CF in Bulgaria vary widely between different sources and, as a consequence, genetic counselling is based on unreliable information where figures from the North-West of Europe (about 1 carrier in 25 individuals in the general population) are used. A pilot screening of newborns for the detection of delF508 carriers was undertaken in the course of the project. The study was based on the fact that delF508 is now known to account for 55% of CF chromosomes, i.e. accurate information on the frequency of delF508 carriers would allow a reliable estimate of the overall frequency of CF carriers. The screening was done by random sampling of the Guthrie cards received in the laboratory for the PKU screening of newborns.
* The preliminary data of the screening (2 000 newborns investigated so far) suggest that in fact the frequency of CF carriers in the overall population of Bulgaria is about 3 times lower than expected. The findings require an increase in the screening sample volume. If confirmed, they will have implication for both genetic counselling (by decreasing the risk of affected offspring for known carriers) and for the strategy of prevention of the disease in Bulgaria.

Inter-ethnic variation in the frequency of CF carriers in Bulgaria

* Among the delF5508 carriers detected during the screening, there is an over-representation of Gypsies and preliminary information suggests that these carriers belong to a specific subset of Gyspies. These findings, if confirmed, are of great significance in terms of the high numbers of expected affected births within this group of Gypsies. This group can therefore be considered to be an important target for primary prevention by carrier detection and prenatal diagnosis.

Population genetics of cystic fibrosis - collaborative studies

* In the course of the project, the laboratory has participated in international collaborative studies of the world-wide population genetics of cystic fibrosis.
* A European study of the chromosomal haplotypes associated with delF508 demonstrated that the mutation has been present in European populations during the Paleolithic period and, in addition, has originated a second time on a chromosomal background different from the ancestral haplotype and has been imported during the Neolithic migration of farmers (publication # 13).
* A world-wide catalogue of CF mutations has been compiled by the International CF Consortium where mutations with a frequency in excess of 3% are represented (publication # 17).

RESULTS
(compare against the set objectives)

* A catalogue of CF mutations in European populations is being set up at the moment where every single mutation has been reported to the coordinating centre, together with information on the geographic origin of the carrier parent.
* Several studies of mutations which are rare world-wide but are found to occur more frequently in specific populations, have been initiated by the Laboratory of Molecular Pathology. These include R347P which is common to Germanic and Slavic populations, 1677delTA which is confined to the Black Sea region and G1069R in combination with L88X (a double mutant allele) which was described in Bulgaria for the first time and is now found in Greek patients as well. Haplotype characteristic have been addressed in each of these studies and the results suggest a common origin of all of these CF alleles (publications #11,19,21).

OBJECTIVE: Designing a strategy for the prevention of cystic fibrosis in Bulgaria

* Two findings obtained in the course of this project have important implications for the strategy of CF prevention in Bulgaria:
    * The extensive heterogeneity of mutations causing cystic fibrosis in Bulgaria.
    * The low overall frequency of carriers and the high frequency within a specific ethnic group.
* Primary prevention of the disease by population-based carrier testing and prenatal diagnosis offered to all detected high-risk couples will be inefficient in terms of cost-benefit as well as in terms of detection rate. However, if the preliminary results on inter-ethnic variation in the frequency of carriers are confirmed, carrier testing in the specific subgroup of Gypsies will be well justified. A project of this nature raises a number of problems related to ethnic discrimination, health education, public perception of genetic prevention etc. These issues are of partiucular complexity given the present-day situation in Eastern Europe and will be addressed in psycho-social studies which will be carried out in advance and will aim at evaluating the feasiblity of targeted prevention of cystic fibrosis.
* Secondary prevention, i.e. prenatal diagnosis of subsequent pregnancies in families where a CF child has already been born, seems to be the justifiable approach for the general population (exluding the above mentioned group of Gypsies).
* The experience gained in the course of the project allows the choice of the following technical approach to the testing of high-risk families:
    * Heteroduplex analysis of exon 10 of the CFTR gene provides information on 63% of CF chromosomes and is fully informative and sufficient in 45% of families;
    * This is followed by SSCP analysis of mutation hot spots, namely exons 21, 11, 7, 17b and 13a (in order of priority), which provides additional information in 17% of CF chromosomes. In combination with the above-mentioned heteroduplex analysis, this stage provides complete information in 65.5% of CF families.
    * Polymorphic analysis including three RFLPs flanking the CFTR gene and three intragenic dinucleotide repeats are to be used in different combinations in families where mutation detection (as described above) does not provide the necessary information. Polymorphic analysis is fully informative in nearly 100% of Bulgarian families.

Part 3

Work plan and time schedule (originally envisaged)

WORK PLAN AND TIME SCHEDULE

* Increasing the number of CF patients and families referred for molecular analysis by stimulating the interest and upgrading the competence of referring clinical centres.
  * Time schedule - the entire duration of the project.

* Choice of the most common mutations to be analysed routinely in all newly referred patients and families.
  * Time schedule - the first year of the project.

* Specifying the conditions of SSCP analysis of the CFTR gene.
  * Time schedule - the first year of the project.

* Routine SSCP analysis in patients where testing for the common mutations has failed to reveal the molecular defect.
  * Time schedule - the entire duration of the project.

* Introducing direct sequencing of the CFTR gene.
  * Time schedule - after the training of a member of the research team at the Department of Molecular Genetics at the Centre for Genetics and Haemotransfusion in Brest, France.

* Routine sequencing of regions of the CFTR gene where sequence variation is suspected on the basis of abnormal SSCP patterns.
  * Time schedule - as above and for the remaining duration of the project.

* Polymorphic analysis of the CFTR gene and of the flanking genomic regions and accumulation of data on mutation/haplotype associations.
  * Time schedule - the entire duration of the project.

* Recording the clinical phenotype of patients in correlation with the mutations in the CFTR gene.
  * Time schedule - the entire duration of the project.

* Developing a strategy for cystic fibrosis prevention.
  * Time schedule - by the end of the project.

* Prenatal diagnosis in high-risk families detecting in the course of the project.
  * Time schedule - the entire duration of the project.

Work plan and time schedule (actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

PROJECT LANDMARKS

- Number of mutations routinely tested for reduced to:
  - 5 mutations - summer 1992
  - 2 mutations - autumn 1993
- Excellent results of SSCP analysis as a result of modifications introduced - Spring 1992
- Direct sequencing working routinely - Summer 1993
- First new mutation detected - January 1993 (in Brest)
- First new mutation detected in the Laboratory of Molecualr Pathology - August 1993
- Microsatellite haplotyping the CFTR gene introduced routinely - Spring 1993
- Heteroduplex analysis of exon 10 on PhastSystem minigels - Autumn 1993
- First stage of pilot carrier testing completed - September 1994

DURATION OF INDIVIDUAL TASKS

EVALUATION CRITERIA

Publications: twenty one.

Services:

- Molecular analysis routinely provided for families affected with cystic fibrosis. A total of 145 families have been analysed. Out of these, 65 have been referred in the course of the project.
- Prenatal diagnosis of cystic fibrosis performed routinely on CVS. 48 prenatal diagnoses provided during the project duration.

Training:

- One member of the research team was trained in DGGE analysis and direct sequencing. The training was provided by the Department of Genetics at the Centre for Genetics and Haemotransfusion in Brest, France.
- Two Honours students have been trained at the Laboratory of Molecualr Pathology. The projects were part of the overall ICGEB funded project and have been successfully completed with first degree Honours and one publication in a refereed journal per student.
- One medical student took part in the research as extracurricular training.
- Two PhD theses are close to completion. The students are part of the research team working on the ICGEB project.
- Two postgraduate training courses for pediatricians and geneticists from genetic counselling units have been organized and have resulted in an increase in the number of new referrals.

NETWORKING

The International Cystic Fibrosis Genetic Analysis Consortium

The Laboratory of Molecular Pathology was one of the founding members of the Consortium prior to receiving ICGEB funding. However, ICGEB funding was vital in making possible the active membership in the Consortium and the contribution of the Laboratory to the international study of cystic fibrosis. Through the Consortium, a number of epidemiological and genotype-phenotype studies have been organized and Bulgarian data have been submitted and have contributed to the representation of southern and, especially of eastern Europe. Membership in the Consortium, apart from ensuring direct access to the most up-to-date priviliged information in the field, has provided a natural framework for the organizing of local studiesd on patients with mutations confined to the specific geographic region.

European Concerted Action for the Coordination of Cystic Fibrosis Research and Therapy

Thanks to the active participation of the Laboratory of Molecular Pathology in the International Consortium and thanks to the publications in the field of cystic fibrosis research, both resulting from the the ICGEB-funded project, we were invited to join the European Concerted Action. A grant proposal was submitted in the summer of 1993 and approved by the European Commission in the beginning of 1994. EU funding for participation in the concerted action will be available over the next three years and will ensure the continuity and completion of the activities initiated as a result of the ICGEB-funded project.

PUBLICATIONS

1. Kalaydjieva L. et al. (1991) CF in Bulgaria, *J Med. Genet.*, 28:807 **2.** Kalaydjieva L., et al. (1991) The Feasibility of CF prevention in Bulgaria, *17th European CF conference*, Kopenhagen, Danmark **3.** Angelicheva D. et al. (1991) CF in Bulgaria, *Annual meeting of the European Society of Human Genetics*, Leuven, Belgium **4.** Angelicheva D. et al. (1991) Three patients with 1677delTA, *8th International Congress of Human Genetics*, Washington, D.C., USA **5.** Osborne L., et al. (1992) Incidence and expression of the N1303K mutation of the CFTR gene, *Hum. Genet.*, 89:653-658 **6.** Savov A.,(1992) High percentage acrylamide gels improve resolution in SSCP analysis, *Nucl. Acid Res.*, 20:6741-6742 **7.** Ferrec at al. (1993) Screening for CFTR gene mutations in a sample of CF patients from Bulgaria, *25th Annual meeting ESHG*, Barcelona, Spain **8.** The Cystic Fibrosis Genotype - Phenotype Consortium (1993) Correlation between genotype and phenotype in cystic fibrosis: Analysis of seven common mutations, *N. Engl. J. Med*, 329: 1308-1313. **9.** Computer prediction in SSCP analysis? (1994) *26-th Annual meeting - European Society of Human Genetics*, Paris, France. **10.** Jordanova A. et al. (1994) Rapid screening method for delF508 carriers. *26-th Annual meeting - European Society of Human Genetics*, Paris, France. **11.** Angelicheva et al (1994) Cystic fibrosis patients from the Black sea region: the 1677del TA mutation. *Human Mutation*, 3: 353-357. **12.** Savov A. et al. (1994) Identification of eight novel mutations in Bulgarian CFTR population. *First Balkan Meeting of Human Genetics*, Thessaloniki, Greece **13.** Morral N. et al. (1994) The origin of the major cystic fibrosis mutation (delF508) in the European populations. *Nature Genet.*,vol 7: 169-175 **14.** Savov A. (1994) Identification of six novel mutations in the CFTR gene of patients from Bulgaria by screening the twenty seven exons and exon/intron boundaries using DGGE and direct DNA sequencing. *Hum. Molec. Genet.*, Vol. 3 No 1: 57-60 **15.** Savov A.,.(1994) G1244V: A novel missence mutation in exon 20 of the CFTR gene in a Bulgarian CF patient., *Hum. Molec. Genet*, Vol 3 No 3: 513-514 **16.** Savov A. et al. (1994) SSCP - a powerfull method for screening of the most common CF mutations in Bulgaria. *Annual meeting of Balkan Clinical Laboratory Society*, Istanbul, Turky. **17.** CF Genetic Analysis Consortium (1994) A population screening report, *Hum. Mut.*, in press. **18.** Angelicheva D. et al. (1994) Meconium ileus in Bulgaria: a molecular study, *Balk. J. of Clin. Lab.*, in press **19.** Savov A. et al. (1994) Triple mutants in Cystic Fibrosis. *Hum. Molec. Genet.* submited **20.** Savov A. et al. (1994) Identification of two novel mutations in the CFTR gene: 2176insC and L571S. *Human Heredity*, submited **21.** Varon R. et al. (1994) Severe cystic fibrosis in patients with R347P mutation, *Human Mut.*,submeted.

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 22 672
2) consumables	US$	2) consumables	US$ 35 618
3) training	US$	3) training	US$ 6 812
4) literature	US$	4) literature	US$ 1 660
5) miscellaneous	US$	5) miscellaneous	US$ 3 238
TOTAL GRANT	**US$.....**	**TOTAL**	**US$** 70 000

Please itemize the following budget categories (if applicable)

Capital equipment

*	DNA Termal Cycler	/Perkin Elmer/
*	Electrophoresis Equipment	/Bio Rad/
*	Slot Blot Aparatus	/Bio Rad/
*	PhastSystem	/Pharmacia/
*	Sequencing Equipment	/Hoefer Scientific Inst./
*	Multiphor II	/Pharmacia/

Training (provide names, duration of training, host laboratory)

* Alexey Savov: 3 months Department of Genetics at the Centre for Genetics and Haemotransfusion in Brest, France
* Participation in international scientific meetings:
  * 8th International Congress of Human Genetics, Washington, D.C., USA
  * 17th European CF conference, Kopenhagen, Danmark
  * Annual meeting of the European Society of Human Genetics, Leuven, Belgium
  * Annual meeting - European Society of Human Genetics, Paris, France.
  * First Balkan Meeting of Human Genetics, Thessaloniki, Greece

Literature

*	American Journal of Human Genetics	1992
*	Journal of Medical Genetics	1992
*	Nature Genetics	1994
*	Human Molecular Genetics	1994

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.

CYSTIC FIBROSIS MUTATIONS FOUND IN BULGARIAN PATIENTS

Chromosome number	Mutation	Exon	Frequency
173	ΔF508	10	59,6
15	N1303K	21	5,1
13	G542X	11	4,5
6	1677 ΔTA	10	2,1
6	R347P	7	2,1
5	R1070Q	17b	1,7
3	2183 A→G	13a	1,0
3	Q220X	6a	1,0
2	2789+5 G→A	intron 14b	0,7
2	3849+10 KB	intron 19	0,7
2	W1282X	20	0,7
2	G85E	3	0,7
2	1717-8	intron10	0,7
2	Y919C	15	0,7
2	G1244E	20	0,7
2	G1069R+L88X	17b+3	0,7
2	2184 insA	13a	0,7
2	N418S	9	0,7
1	1898+3A→G	12	0,3
1	Q493R	10	0,3
1	L571S	12	0,3
1	2176 insC	13a	0,3
1	G1244V+S912L	20+15	0,3
1	Q2X+R3W	1	0,3
40	unknown		13,8