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Meetings of the Molecular Medicine Study Group Academic Year 1997-1998

Under the auspices of the Life Sciences and Biotechnology Network of the Malta Council of Science and Technology

The following collection of abstracts is the record of the meetings of the Molecular Medicine Study Group held in the 1997-1998 academic year under the auspices of the Life Sciences and Biotechnology Network of the Malta Council of Science and Technology.

The group first met in 1990, initially as the Haematology Study Group under the leadership of Professors John Rizzo Naudi and Alex Felice. The intention was to link the new research programmes on thalassaemia and haemophilia at the University of Malta with the needs of the patients, their families and others at risk. Subsequently, as the molecular genetics programmes expanded the group started to consider issues raised by the new work involving other gene systems as well.

The goal of the Molecular Medicine Study Group is to discuss progress in advanced molecular biology and genetics with regard to the developments taking place at the University of Malta and elsewhere and to review applications of these new technologies in the hospital and clinics, with due consideration being given to organisational, technical and ethical issues.

The group meets on the last Thursday of each month at the Medical School. The meetings take the format of a brief presentation followed by a discussion. Those who have expertise or are working in the field of molecular sciences and those who are interested in clinical applications are invited to participate. Anyone who would like to be put on the mailing list is asked to contact Ms. Dorita Galea on telephone 3290-2917 or email dagi1@um.edu.mt

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Clinical and Molecular Pathology of the ß+IVSI-6C Thalassaemia in Malta

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The main objective of this study was to characterise the ß-thalassaemia mutations present in Malta, evaluate treatment protocols, study the correlation between the genotypical and phenotypical pictures, evaluate the criteria for the proper identification of thalassaemia heterozygotes and investigate the causes for the bone disease in thalassaemia homozygotes.

Data on the molecular defects leading to ß-thalassaemia were obtained from 28 homozygotes out of the known 29 subjects. Four different mutations were encountered, with the ß$^+$IVSI-6(T→C) accounting for 71.4% of all ß-thal alleles [ß$^+$IVSI-110(G→A) = 12.5 %; ß^{0}IVSII-1(G→A) = 10.7 %; ß^{0}Codon 39(C→T) = 5.4%]. The ß$^+$IVSI-6(C) allele was present on both haplotype VI and VII while the ß^{0}Codon 39(T) and the ß$^+$IVSI-110(A) were associated with haplotypes I and IX respectively. The ß^{0}IVSII-I(A) mutation was found within haplotypes III except in one case that had an unusual VI/III hybrid haplotype. The VI/III hybrid haplotype was characterised by a low HbF (7.7%) in contrast to the other 5 cases that had a high HbF (~60%). Data collected prospectively on the ß$^+$IVSI-6(C) homozygote children, indicated that the disease presented as a moderate to severe condition needing regular blood transfusion (mean = 70 ml/kg/year) for normal growth and development. Splenectomy had little effect on the blood transfusion requirement of the ß$^+$IVSI-6(C) homozygotes. On the other hand, the adult ß$^+$IVSI-6(C) homozygous condition was characterised by a mild disease (mean Hb = 8.2g/dl) with only occasional transfusions. Intra-allelic heterogeneity in the level of HbF was observed among the ß$^+$IVSI-6(C) homozygotes and these could be divided into two groups, one with a relatively high HbF (mean = 15.0%) and one with a low HbF (mean = 4.5%). Statistically significant ($p<0.05$) gender differences in the level of HbF was also observed with female patients having in general, a higher HbF level.

The high incidence of a relatively mild mutation and the presence of iron deficiency amongst the population, posed problems in the proper identification of ß-thalassaemia heterozygotes. Indeed 37% (N=19) of obligate ß$^+$IVSI-6(C) heterozygotes had an MCV<80fl and a HbA_2 between 3.0 and 3.5%. In an attempt to improve in the identification of the ß+IVSI-6(C) heterozygotes, while keeping the cost of population testing to a minimum, a new approach in the identification of individuals at risk was evaluated with a computed index (10 x RBC^3 x HbA_2/Hb^3). The exclusion of iron deficiency was further considered with a revised discriminant/cut-off value for serum ferritin, which was higher than that employed so far. A definite diagnosis amongst those individuals deemed at risk would be obtained by DNA analysis for the prevalent mutation within the population.

Osteopenia as documented by a low bone mineral density using DEXA, was present in the majority of the homozygote subjects and was apparent as early as 5

years of age, despite a seemingly adequate hypertransfusion regime. In an attempt to elucidate possible causes for the observed osteopenia, the level of bone biochemical markers for bone formation (serum procollagen I carboxyterminal propeptide and osteocalcin) and for bone turnover (urinary deoxypyridinoline crosslinks and serum tartrate resistant acid phosphatase) were measured amongst the thalassaemia patients. The low serum osteocalcin level accompanied by normal levels for urinary deoxypyridinoline crosslinks and serum procollagen I carboxyterminal propeptide indicated that lack of proper mineralisation could result in the osteopenia observed within this group of patients. Nutritional deficiencies associated with low body weight might be possible causes of the lack of mineralisation among the patients with 'mild' alleles.

Classification of von Willebrand Disease

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Von Willebrand Factor (vWF) plays a key role in platelet adhesion; it also acts as a carrier for coagulation Factor VIII (FVIII). von Willebrand Disease (vWD), the most common bleeding disorder, is caused by a deficiency in vWF.

The most recent revised classification of vWD distinguishes partial quantitative (Type 1), qualitative (Type 2) and total quantitative (Type 3) deficiency of vWF. The goal for the new classification is to maximize the correlation of subtypes with the treatment choices as it depends on the accurate diagnosis and classification of vWD in the patient.

The main aim of this study is to classify known Maltese vWD patients and their family members according to the newly revised classification. There were two new assays performed. vWF:Ag by *Enzyme Immuno Assay* method and vWF Multimers by SDS-Agarose Electrophoresis, blotting to nitrocellulose membrane and staining by a sensitive peroxidase method.

The results showed that, 17 patients (4 families) carried the vWD, 14 patients (3 families) of which were Type 1 and 3 (1 family) were Type 2B.

Molecular Pathology of Infantile GM1 gangliosidosis

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The lysozomal storage disorder G_{M1} gangliosidosis is a genetic neurological disorder caused by a complete or partial deficiency of acid ß-galactosidase. It is usually classified as being of infantile, juvenile or adult forms. Infantile GM_1 gangliosidosis is relatively common in the Maltese population, with a heterozygous incidence of 3.3% and 0.027% homozygotes. The molecular lesion associated with this pathology was studied in two unrelated Maltese families. Fragments containing all the exons and flanking regions of the ß-galactosidase gene, were amplified using Polymerase Chain Reaction (PCR) and then sequenced. A variant was identified and denoted Spl 7. This double point mutation, CA→GT, lies within the IVS 7 of ß-galactosidase gene, at position 9 and 10 bp downstream of the 3' end of exon 7 (the 3'end is at cDNA position 842). *Taq* I restriction digestion, confirmed this mutation. In addition *Bfa* I restriction enzyme digestion also confirmed the presence of the two mutations in *cis*. This variant was confirmed to be non-polymorphic by *Taq* I digestion of a 100 random chromosomes. Other variants are present in the Maltese population, but they were not characterized in this study. Urinary oligosaccharide analysis was used to test the two Maltese families for the presence of a high amount of oligosaccharides. The result was consistent with the molecular study.

Ethical issues connected with the privacy of persons who are found to have genetic defects

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Information on genetic defects constitutes knowledge of a very intimate nature and can change a person's life radically. Knowledge of one's own genetic defects can be a source of great anxiety even if this information is kept secret.

When the confidentiality of this information is broken, the person is often placed in a very vulnerable position and his/her basic rights are threatened. Very serious harm can be done to the person involved.

On the other hand genetic information pertaining to one person can have serious implications for others' welfare including spouses, children, and extended family members.

How ought a genetic researcher/doctor deal with the information of genetic defects of his/her patients?

Update on Huntington's Disease: Recent research offers new hope for future therapy

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The genetic abnormality underlying Huntington's disease (HD) is that the $(CAG)_n$ trinucleotide repeats, which occur normally at the huntington gene locus 4p16, become unstable and amplified when transmitted from one generation to the next. The gene product, huntingtin contains polyglutamine repeats in its exon 1 region. Huntingtin is essential for neurogenesis and is expressed in the cytoplasm of neurons in most parts of the brain including the basal ganglia.

New insights have been obtained from animal and test-tube models. Transgenic mice having 115 to 165 CAG repeats of the human HD gene inserted into their genome develop abnormal movement disorders and manifest

nuclear intra-neuronal inclusions with strong immunoreactivity for huntingtin. Biopsies of cerebral cortex and caudate nucleus of HD patients in 1979 had demonstrated similar inclusions but the study included a small number of subjects and was not repeated for ethical reasons.

In the test tube model, a fusion protein of huntingtin containing polyglutamine expansions and glutathione S transferase was produced in genetically engineered *E. coli*. When the polyglutamine region of the recombinant protein was cleaved with proteolytic enzymes, it formed high molecular weight aggregates with fibrillar morphology similar to the intra-nuclear aggregates of transgenic mice. These findings suggested that a similar mechanism of cleavage and fibrillar deposition might be operative in human neurons in HD.

There is evidence that intra-neuronal aggregates also form in other diseases with $(CAG)_n$/polyglutamine expansions including dentato-rubral-pallido-luysian atrophy, spinocerebellar ataxia types 1,2,3 and 6, and spinal-bulbar muscular atrophy. Scrapie prions and Alzheimer's disease also produce protein aggregates with fibrillar morphology.

The suggested mechanism of action in Huntington's disease is that huntingtin is cleaved in the cytoplasm and if the polyglutamine part reaches a critical size it aggregates into clumps which migrate into the nucleus of neurons causing them to degenerate.

The test tube model of huntingtin opens new prospects of finding new drugs that would inhibit the formation of aggregates. It is hoped that the test process can be automated for testing thousands of possible drugs. Candidate drugs can then be tested on the transgenic mice models.

In vitro expression of new mutant coagulation factor VII proteins

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Pre-operative coagulation studies on a 4-year old girl indicated the presence of coagulation factor VII deficiency in a kindred from Malta. Factor VII assays with a panel of thromboplastins from four different species, and factor VII antigen assays, indicated phenotypic heterogeneity within the kindred. Sequence analysis of the factor VII coding region and 5' untranslated region demonstrated the presence of two new missense mutations. One of these was a cytosine to adenine transversion at position 8,906. This mutation, designated factor VII Malta I, leads to the substitution of the proline at position 134 by threonine. The other mutation was cytosine to thymine transition at position 10,648. This mutation, designated factor VII Malta II, leads to the substitution of the alanine at position 244 by valine. Sequence analysis also showed that factor VII alleles of the kindred were of two different frameworks, framework 1 and framework 2. Framework 2 is known to be associated with decreased levels of factor VII coagulant activity, factor VII antigen and activated factor VII. The factor VII Malta I mutation occurred on a framework 1 allele whereas the factor VII Malta II mutation occurred on a framework 2 allele. In all, four factor VII alleles segregated in the kindred. These were: (i) a wild-type framework 1 allele, (ii) a wild-type framework 2 allele, (iii) a framework 1-factor VII Malta I allele, and (iv) a framework 2-factor VII Malta II allele. These four alleles resulted in a complex pattern of genotypic combinations, which accounted for the phenotypic heterogeneity among members of the kindred. Both the factor VII Malta I and the factor VII Malta II mutations were likely to be associated with decrease in cross-reacting material.

In order to investigate the effect of the two amino acid substitutions on the framework 1 gene product, framework 1 factor VII cDNAs coding for factor VII Malta I and factor VII Malta II were constructed and expressed in Chinese hamster ovary cells. The specific activity of the factor VII Malta I recombinant variant did not differ significantly from that of recombinant wild-type factor VII expressed by the wild-type framework 1 factor VII cDNA. On the one hand, the factor VII Malta II recombinant variant had a specific activity slightly decreased as compared to that of the recombinant wild-type protein. Both variants were fully activated by activated factor X. Furthermore, the K_{cat} and K_M for activation of factor X in the presence of tissue factor, by activated recombinant factor VII Malta I and by activated recombinant factor VII Malta II, did not differ significantly from those by activated wild-type factor VII.

The data obtained excluded gross dysfunctional effects of the two amino acid substitutions on the functional characteristics of the framework 1 gene product. However, the studies done to date suggest a complex defect perhaps due to a small decrease in activity, defective secretion, decreased stability, or sequestration in an inactive complex in plasma.

Reference:

Alshinawi C, Scerri C, Galdies R, Aquilina A, and Felice AE. Two new missense mutations (P134T and A244V) in coagulation factor VII gene. *Human Mutations* 1998; **1**: S189-S191.

Genotype-Phenotype Correlation and Predictive Medicine in Cancer. A brief report on some topics discussed at the 2nd Gaslini-IARC Course in Cancer Genetics held in Sestri Levante (Genoa) in September 1997

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With improvements in genetic diagnostics and with the establishment of reliable genotype-phenotype relationships, medicine is entering a new era. One field where predictive medicine has a lot of potential is that of cancer. Familial Medullary Thyroid Carcinoma (FMTC) is one model for predictive medicine in this field. MTC accounts for less than 10% of thyroid cancers. One third of the cases occur in high risk families as part of an autosomal dominant inherited cancer syndrome: multiple endocrine neoplasia type (MEN 2). There are three variants of MEN2 known as MEN2A, MEN2B and FMTC. Different mutations in the RET proto-oncogene

are responsible for the different phenotypes. The RET protein is a transmembrane tyrosine kinase receptor that dimerizes after ligand activation triggering intrinsic enzyme function and autophosphorylation. The activated receptor can then bind and/or phosphorylate other molecules that work as intracellular transducers. Point mutations in one of 5 cysteines clustered in the extracytoplasmic domain (codons 609, 611, 618, 620 in exon 10 and 634 in exon 11) of RET were found in 97% of patients with MEN2A and 86% of patients with isolated FMTC. All these mutations result in ligand independent activation of RET. On the other hand MEN2B is almost always the result of a unique point mutation, often *de novo*, in the tyrosine kinase domain of codon 918 that substitutes a methionine for a threonine. The mechanism of the transforming activity is not yet clear.

Another cancer where genotype-phenotype relationships are being established is familial adenomatous polyposis (FAP). FAP is caused by germline mutations in the adenomatous polyposis coli (APC) gene. The gene is 8550 bp long and has 15 exons that code for a 312 kDa protein presumably involved in cell-cell interactions and in the intracellular transmission of cell adhesion signals. It has been shown that chain-terminating mutations located close to the 5' end of the APC gene result in a generally mild and variable FAP phenotype called attenuated adenomatous polyposis coli (AAPC). AAPC is generally characterized by fewer polyps (≤100) and delayed age of onset (approximately 10 to 15 years later than those observed in patients with a more classical FAP). The lifetime risk for colorectal cancer remains unchanged. Chain-terminating mutations located between codon 200 (exons 5 to 6) and 1600 of the APC gene usually result in higher densities of colonic tumours. Chain terminating mutations in the region between codons 1250 and 1464 are invariably associated with a profuse phenotype with up to 5000 colorectal adenomatous polyps. Mutations outside this region and downstream of codon 213 and upstream of codon 1597 lead to a sparse FAP phenotype with patients developing between 1000 and 2000 colorectal polyps. Even occurrence of congenital hypertrophy of the retinal pigmental epithelium (CHRPE) and hereditary desmoid disease was found to be associated with the site of the mutation. However, it must be stated that independent studies have given inconsistent results. This may be due to the effect of modifier genes and/or environmental factors. More precise genotype-phenotype correlation will eventually lead to preventive genetic counselling for individuals at risk.

A very important topic in this field is breast cancer, the most common cancer among women in the Western world. About 5 to 10% of these cases are inherited in a dominant autosomal manner. Mutations in the BRCA1 gene account for approximately 40% of families with at least 4 members with breast cancer under 60 years of age and for over 80% of families with histories of both breast and ovarian cancer. On the other hand mutations in BRCA2 have a lower risk of association with ovarian cancer than BRCA1 but it has a higher risk of association with male breast cancer. The lifetime risk for breast cancer in women who carry a BRCA1 mutation is 85%; the lifetime ovarian cancer risk is 60%. The risk of developing a second primary breast cancer after the first is about 65%.

Further studies on cancer mutations and collection of data that relate genotype to phenotype will allow more definite associations to be made enabling more preventive measures to be utilised.