NUCLEOTIDE POLYMORPHISM IN SCHIZOPHRENIA

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DECLARATION

I hereby declare that the thesis entitled "NUCLEOTIDE POLYMORPHISM IN SCHIZOPHRENIA" submitted for Ph.D degree at the university of Pune has not been submitted by me for a degree at any other university.

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ABBREVIATIONS

bp:	Base pair
dATP:	deoxyadenosine 5' triphosphate
dCTP:	deoxycytidine 5' triphosphate
dGTP:	deoxyguanosine 5' triphosphate
dTTP:	deoxythymidine 5' triphosphate
DNA:	deoxyribonucleic acid
IAA:	iso – amyl alcohol
Kb:	Kilo base pairs
µg:	micro gram
µl:	microliter
ml:	milliliter
µM:	micromolar
mM:	millimolar
M:	molar
ng:	nanogram
PAGE:	polyacrylamide gel electrophoresis
µCi:	micro curie
PCR:	polymerase chain reaction
Min:	minute
mRNA:	messenger ribonucleic acid

rpm:	revolutions per minute
SDS:	sodium dodecyl sulphate
TE:	Tris – EDTA buffer
U:	units of enzyme
CT:	computerised tomography
MRI:	magnetic resonance imaging
RBC:	red blood cells
PBMCs:	peripheral blood mononuclear cells
PBS:	phosphate buffered saline
BDNF:	brain derived nerve factor
NGF:	neuronal growth factor
RED:	repeat expansion detection
RT:	room temperature
°C:	degree centigrade

Chapter 1

SCHIZOPHRENIA: NATURAL HISTORY AND GENETIC ASPECTS

INTRODUCTION

Brain is the controlling centre of an organism's body and behaviour. *Homo sapiens* are considered as the most evolved species as they have the most evolved brain, and consequently highest social activities and mental illness. Mental illness profoundly affects an individual's capacity to think, feel and act, causing severe constrains on individual's activities, the consequences of which are only confined to the individual alone but have reflections on the society also. In the United States, the annual costs for patient treatment of mental, social service, disability payments, lost productivity and premature mortality, and to family amount to more than $150 billion (NIH 1995).

Amongst the psychotic illnesses, schizophrenia is the most devastating one striking primarily teenagers and young adults, leading to severe psychological, social, and vocational disability during the potentially most creative and productive years of their life. Aetiology of schizophrenia is speculative, and its pathophysiology and psychopathology are complex and heterogeneous. Despite strong evidence for genetic susceptibility, no specific gene(s) have been unambiguously identified in schizophrenia. Possible involvement of multiple susceptible genes has been hypothesized each of which may contribute to the pathophysiology, and influence this complex disorder. However, mere presence of a given susceptible gene(s) may not result in the expression of the disorder, as many researchers believe that environment plays a critical role towards modulating genetic expression of susceptible gene(s) in schizophrenia.

Tracking disease-related genes successfully is feasible if a population of individuals with the genetic variant, and techniques for identifying specific genes are available. Ideally, the definition of a disease should be clear enough so that all the diagnosed individuals have relatively uniform symptoms; thereby increasing the chance of identifying affected individuals unambiguously. For example, In case of sickle cell anaemia, specific deformation of the blood cells is indicative of the disease. However, this is not the case for schizophrenia as no clear-cut tests have been defined to identify the disease. Nonetheless, much progress has been made over the years and researchers have developed procedures to permit a fairly reliable diagnosis of schizophrenia.

In humans, only 35% of the DNA is suggested to be responsible for coding and there are a little more than 35,000 functional genes. Inheritable susceptibility to various diseases is expressed when a particular gene fails to give correct instructions for a specific trait. Gene identification is carried out by constructing fine maps of known gene locations and functions or by comparing the DNA of affected and non-affected individuals.

The general experimental strategy of identifying the gene(s) responsible for the disease, in the first phase, is to identify the vulnerable gene(s). The work plan, therefore, is to choose a set of candidate genes such as those likely to be involved in the disease pathophysiology and study their association in affected individuals and matched controls.

Identification of specific gene(s) associated with the disorder holds a great promise for advancing diagnosis and treatment. Even if there are multiple genes responsible for the disorder,

intervention even at a single gene level may be useful for prevention or treatment. Finally, predictive screening will greatly increase the feasibility of prevention of the disorder, as interventions can be tailored for and provided to individuals having a high risk of developing the disorder.

SCHIZOPHRENIA THE DISORDER

Schizophrenia is the most devastating psychotic illness as it affects individuals in their early life and continues for the rest of the life. At the beginning of the twentieth century, the founders of modern psychiatry believed schizophrenia to be a hereditary disorder. Attempts to study the genetics of schizophrenia began in 1920 by Luxemburg and Rudin (Stromgen, 1994). However, after 1945 with the dominance of psychoanalytic theory in America and disenchantment with the genetic theory in Europe, the genetic or the biological basis of schizophrenia was widely challenged to the extent that R.D. Laing described schizophrenia as 'a sane response to an insane world'.

Pioneering work of Emil Kraepelin in Germany and Eugene Bleuler in Switzerland improved criteria for diagnosis of schizophrenia. Kraepelin called it 'dementia praecox'. Because of it's early age of onset, which further followed a progressive course of mental decline, Bleurer dropped the term dementia praecox as he came across individuals who succumbed to the disease in adulthood and also occasional cases remitted. He concluded that schizophrenia is a group of closely related diseases not characterized by deterioration of intellect but by a splitting of cognitive sides of personality from the affective or emotional side and hence, called the disease schizophrenia or a splitting of the mind.

The current criteria for identifying schizophrenia (DSM IV and ICD III) (American Psychiatry Association, 1994) require that the patient be continuously ill for six months and that one or more of the following groups of symptoms be present:

1. Bizarre dilution: of being persecuted or having ones feelings, thoughts, and action controlled by outside force.
2. Auditory hallucinations: hearing voices commenting on ones action.
3. Disorder of thought, consisting of incoherence, loss of normal association between ideas, or marked poverty of speech accompanied by loss of emotional responsiveness or less specific delusions and hallucinations.

Schizophrenia is defined as characteristic but non-specific disturbances in form and content of thought, perception, emotion, and sense of self, volition, social relationship, and psychomotor behaviour.

SCHIZOPHRENIA SUBTYPES AND ASSOCIATED PROBLEMS

Schizophrenia sub typing has a long history. Earlier, distinctions were made on the basis of phenomenology, aetiology or prognosis, namely,

- Phenomenology - hallucinatory, hebephrenic, catatonic and simplex forms
- Aetiology - reactive (psychogenic) / process, organic/endogenous forms
- Prognosis – benign /non-benign, process/non-process, deficit/non-deficit, good prognosis/ poor prognosis.

Later, distinctions involved a combination of several disease characteristics - onset, symptoms course, response to treatment and biological parameters (ventricular enlargement).

Type I and type II schizophrenia *(Crow, 1980)*

Type I was characterized by acute onset, normal brain structure, normal intellectual function, good response to antipsychotic drugs, possibly increased dopamine D2 receptors, and absence of negative symptoms.

Type II was characterized by insidious onset enlarged cerebral ventricles, intellectual deterioration, poor response to antipsychotic drugs and prominent negative symptoms.

Positive and negative schizophrenia. *(Andreasen, 1982)*

The prominence of positive symptoms with minimum or no negative symptoms characterized positive schizophrenia.

The prominence of negative symptoms with minimum or no positive symptoms defined negative schizophrenia.

Positive, negative and mixed types*. (Andreasen, 1982)*

Patients who failed to meet the criteria for both positive and negative schizophrenia or had a substantial number of both sets of features were classified as mixed.

Deficit and non-deficit schizophrenia. *(Carpenter, 1988)*

Patients with primary, enduring negative (deficit) symptoms, carefully distinguished from transient negative symptoms secondary to other factors, were labelled as the deficit group, and those without the same were the non-deficit group.

There have been problems with sub typing. Pure positive or negative phenomena, on which most of the distinctions were made, are rarely observed. These are not longitudinally

independent phenomena, but are found to be interchangeable during the course of schizophrenia. It is thought that deficit symptoms exist only in a subgroup of schizophrenic patients and thus may provide the basis for categorization (Kirkpatrick, 2001). However, this needs to be proved.

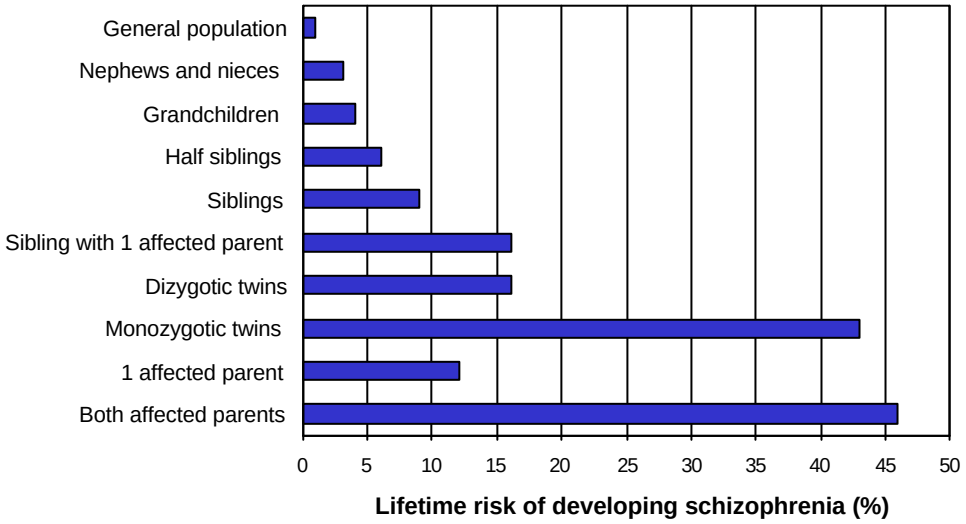
EPIDEMIOLOGY AND MODE OF INHERITANCE

Due to the problems in case definition and determining the date of onset of the illness, the true incidence of schizophrenia in a population is difficult to obtain. Most incidence rates are based on treated incidence, generally targeted to the size of population over a specified period. The number of affected persons markedly increases if schizophrenia spectrum disorders are included in prevalence estimate. No large difference is seen in the manifestation rate of schizophrenia across cultures and geographical areas. This, however, does not exclude the possibility that pockets of usually high or low incidence maybe found in different populations, e.g. the morbid risk of 2.66% calculated by Book, in 1953 in a population isolated in northern Sweden.

Reported variations in prevalence rates of schizophrenia are much greater than variations in incidence rates as differential mortality, migration, and other demographic factors affect them. Hence, incidence rates (number of new cases occurring in a given population over a specified period) are better suited than prevalence (number of affected individuals in a given population) rates for comparisons between different populations. In general treated prevalence rate for schizophrenia is higher for males then females.

Schizophrenia shows familial aggregation. For example, close relatives of a person with a disorder are at an increased risk for the disorder as compared to a person chosen at random from the general population. In accordance with the WHO international study, the lifetime risk for schizophrenia in the general population is 1% irrespective of race of origin or geographic location (Jablensky *et al,* 1991). Some studies have reported a greater risk in men (Kendler and Walsh, 1995) and the risk are ten times higher in siblings of schizophrenics and increases with the number of relatives already affected. When one of the parent and a sibling are affected, the risk is 16%, highest being 46% when both parents are affected. However, it is clear from Table 1 that the risk of schizophrenia in different classes of relatives does not confirm with those predicted by a simple Mendelian pattern of transmission.

Table 1: Average risk of developing schizophrenia.



Conclusions drawn from the family studies of schizophrenia carried out over a period between 1980 -1992 indicate that though the risk for schizophrenia in first-degree relatives of schizophrenic probands varies widely across studies (1. 4% - 8. 9%) it is higher than the general population. Differences in diagnostic criteria or statistical fluctuations in small samples can probably explain much of the fluctuations, as the lowest risk is found in the smallest study.

The risk for schizophrenia in the relatives of nonpsychiatric control probands is relatively similar across studies (0.2% - 1.1%), which corresponds closely to the range of risk for schizophrenia found in the general population. In every study, carried out so far the risk for schizophrenia was higher in the relatives of schizophrenic proband than in the relatives of control proband. The correlation in liability for all studies fell in a relatively narrow range from 0.23 - 0.41 indicating a strong degree of familial aggregation.

AETIOLOGY AND PRINCIPAL HYPOTHESES FOR SCHIZOPHRENIA

As of today, the causes of schizophrenia are unknown. Though genetic factors are found to play an important role, it is not clear which genes are involved, whether the same or different genes are involved in all cases, or how they contribute to the aetiopathology. Various environmental factors like stress, gestational and birth complications, viral infection are also thought to be involved in the causal pathway of schizophrenia. Principal hypotheses regarding the cause of schizophrenia include.

1. Neurodevelopmental hypothesis.
2. Genetic hypothesis.

Neurodevelopmental hypothesis

This hypothesis suggests that a brain 'lesion' is present or is acquired early in life but does not fully manifest itself until early childhood or late adolescence (Weinberger *et al,* 1987; 1995). Whether schizophrenic condition arises due to the failure of the brain to develop normally or due to a disease process that alters a normally developed brain was a debatable question. However, preponderance of evidence is consistent with the hypothesis that schizophrenia is a neurodevelopment disorder. Developmental process of cell growth, cell migration, differentiation, axon connection, and long-term neuron survival follow a cascade of reactions. Establishment of a normal brain cytoarchitecture occurs if the above-mentioned development takes place flawlessly. For efficient functioning, elimination of certain nerve cells and synaptic connections occur during adolescence. Inefficient pruning of nerve cells and synapses or errors in selection for pruning could underline dysfunction, which may later lead to schizophrenia symptoms.

Factors affecting neurodevelopmental hypothesis

Birth and pregnancy complication

Over the last three decades, several studies have found that obstetric complications may be a risk factor for the late development of schizophrenia. (Woerner *et al*, 1973; Reddy *et al*, 1989; Gunther *et al*, 1994; Rifkin *et al*, 1994). It has been speculated that obstetric complications may lead to abnormal neural development (Mednick and Cannon, 1991). Alternatively, a propensity to a difficult birth could be secondary to a genetic susceptibility towards schizophrenia, in which case obstetric complications would lie on the same pathway and may have no

independent aetiology /role. The proposed mechanism is diminished oxygen supply or hypoxia resulting from pregnancy and birth complications. Secondly, the brain regions that are most frequently implicated as deviant in schizophrenia namely hippocampus, a component of the limbic system, the cerebral cortex and basal ganglia, are among the areas in the developing brain that are most sensitive to adverse effects of hypoxia. A meta-analysis designed to analyse the uniformity of the findings across studies performed thus far by Geddes and Lawrie showed that the pooled odd ratio of the effect of exposure to obstetric complications on the subsequent development of schizophrenia was 2.0 (95% CI 1 6- 2.4) (Geddes and Lawrie, 1995). That is individuals exposed to obstetric complications are approximately twice as likely as controls to subsequently develop schizophrenia.

Viral infections

Viral theories for the aetiology of schizophrenia remain popular despite the difficulty in validating any particular version. They have the power to explain the localization of pathology that is necessary to account for a range of manifestations in schizophrenia without requiring extensive infection. Viral infections of the central nervous system during the prenatal or perinatal period may contribute to the increased risk of subsequent schizophrenia. Data from a Finish study showed that children who had infections of the central nervous system had a five fold greater risk of developing schizophrenia than did the comparison children. It is envisaged that viruses with an affinity for the CNS cause alterations in the functioning of the involved nervous system (Oldstone *et al*, 1989; de le Torrey *et al*, 1983) or the by-products of viral infection have a toxic effect on the nerve

functioning. Alternatively, viral infection itself could cause neurodevelopment errors during the second trimester of pregnancy. To name a few cytomegalo virus (Torrey *et al*, 1983) reovirus (Averback, 1982) influenza (Menninger, 1926; 1928) and retrovirus (Crow, 1984; 1986) are some of the viruses implicated in the pathophysiology of schizophrenia. Post-mortem studies, however, show lack of physical signs of active viral infection.

An autoimmune pathology could result when the virus induces the host to fail to recognize it's own tissue as self and subsequently mount a destructive immune response. Various parameters such as elevated serum immunoglobulin levels, decreased mitogen response, morphologically abnormal lymphocytes, increased antibody to nuclear factor, increased anticytoplasmic antibodies, increased serum IL-2 receptor concentration, increased serum IL-6 concentration, and decreased CD-4 + T cells in schizophrenia formed the basis for this hypothesis (reviewed by Ganguli, 1994). These abnormalities may also be due to antipsychotic medication, prolonged duration of illness, clinical state, nutritional status, and substance abuse.

Neuropathophysiology

Computerized tomography (CT) scans of brains of schizophrenic patients provided the first definite proof of brain pathology (Johnstone *et al*, 1976). Dilated lateral ventricles were reported in these patients that were confirmed using CT scan and magnetic resonance imaging (MRI).

Morphological evidences through neuroimaging studies

Enlarged ventricles, particularly the lateral and third, is the most robust observation of neuroimaging studies. This abnormality is

characteristic of the entire schizophrenic population, rather than a subgroup (Daniel *et al*, 1991). It is observed in never-medicated patients, at the onset of psychosis (Degreef *et al*, 1992; Lim *et al*, 1996; Gur *et al*, 1998; Whitworth *et al*, 1998; Zipursky *et al*, 1998) and is not a result of medication or illness chronicity.

Other observations include reduced cortical volume, wherein grey matter reduction is more than that observed with white matter (Lawrie and Abukmeil, 1998; Zipursky, 1998) and reduction in the volume of a number of brain regions. Reduction in temporal lobe volume (Barta *et al*, 1990; Schlaepfer *et al*, 1994; Zipursky *et al*, 1994; Flaum *et al*, 1995; Menon *et al*, 1995; Tune *et al*, 1996; Hajek *et al*, 1997; Marsh *et al*, 1997; Reite *et al*, 1997; Sullivan *et al*, 1998) is most prominent while frontal, parietal and occipital lobes are also reported to be affected (Andreasen *et al*, 1994; Bilder *et al*, 1994; Schlaepfer *et al*, 1994; Zipursky *et al*, 1994). In addition to cortical regions, a number of sub cortical structures, namely, thalamus, corpus callosum, basal ganglia, cerebellum, cavum septum pellucidi are also found to be affected.

Post-mortem studies of brains of schizophrenic patients have reported a decrease in brain weight (Brown *et al*, 1986; Pakkenberg *et al*, 1987; Bruton *et al*, 1990), brain length (Bruton *et al*, 1990) and volume of cerebral hemispheres (Pakkenberg *et al*, 1987). Neuronal atrophy with unchanged cell numbers has also been observed (Rajkowska *et al*, 1998; Selemon *et al*, 1995). It is likely that the neurodevelopment deficit is related to reduced proliferation and migration contributing to the above-mentioned changes.

From the above studies, it appears that the proposed lesion in schizophrenia, which is a necessary condition for the development of the syndrome, must exist in combination with other factors to clinically manifest as a disorder. Post-mortem studies are not indicative of any ongoing neuropathological process, such as reactive gliosis, inclusion bodies, or inflammation. The findings suggest either an old episode of brain damage, or a displastic condition. Hence, several investigators have interpreted their findings as indicative of a congenital lesion (Jakob *et al*, 1986; Kovelman *et al*, 1984; Benes *et al*, 1986). Data from CT scan studies also suggest that the lesion is old and inactive. Ventricular enlargement is reported in the first episode schizophreniform patients (Weinberger *et al*, 1982). The observation that size correlates with poor premorbid social adjustment (Weinberger *et al*, 1980; Williams *et al*, 1985) and reports of a link between ventricular enlargement and a history of prenatal complications (Williams *et al*, 985; Turner *et al*, 1986) also suggest that the pathology exists early in life.

Abnormal brain development in schizophrenia

An important factor that influences the clinical manifestations of any lesion is the age of the brain. The lesion itself is static, but it's effect on the neurological function changes. If a lesion affects a brain structure or region that has yet to mature functionally, the effects of the lesion may remain silent until that structure or system matures (Adams *et al*, 1982). The existence of a critical period of vulnerability for expression of schizophreniform psychosis suggests that neural systems mediating this behaviour reach a functional peak during this period. Even a static lesion that remains through out life might be linked to and depends on

the critical phase of brain maturation. Hence, it follows from the considerations that a lesion in this neural system could remain clinically silent or not be expressed as psychosis until early adulthood.

One of the brain areas implicated in schizophrenia that appears to reach functional maturity in early adult hood is the dorsolateral prefrontal cortex (DLPFC). In humans, the DLPFC is the last brain area to begin myelination and may be the only brain area that continues to undergo myelination throughout life (Yakovlev and Lecours, 1964). Though the meaning of this in terms of behaviour is unclear, it suggests that this area remains relatively plastic or uncommitted until adulthood. Synaptic pruning or the normal developmental regressions of excessive inter neuronal contacts does not level off until puberty. It might be predicted that since the DLPFC reaches functional maturity relatively late in life, a lesion of the DLPFC could have little impact on the behaviour during childhood. Experiments in monkeys support this assumption where a DLPFC lesion does not dramatically impair the behaviour of infant or prepubescent monkeys, and yet it devastates the capacity of the adult monkeys to perform delayed- response tasks (Goldman *et al*, 1977). Further, reports that some monkeys with infantile DLPFC lesions do well on delayed response tasks during childhood lose their ability to perform when they reach adulthood are even more striking (Tucker *et al*, 1967); Goldman *et al*, 1971).

The above experiments suggest that DLPFC function is not rate limiting for many behaviours until adulthood (Goldman *et al*, 1971) and that a lesion in the DLPFC can be consistently compensated for only before this stage of life. It is, however, an

over simplification to assume that such a lesion in humans would be totally clinically silent before adolescence. It would probably be associated with subtle behavioural abnormalities, social awkwardness, and shyness. This might explain the tendency for the premorbid history of patients with schizophrenia to be characterized by such traits (Kraepelin, 1971; Bleuler, 1950; Fish, 1977).

There is considerable clinical and animal research data indicating that the time at which a brain lesion occurs is not necessarily the time for clinical manifestation. Since the symptoms of schizophrenia may be linked to the dysfunction of the neural systems that normally reach physiological maturity in late adolescence and early adulthood, the clinical onset of the illness maybe a function of this normal maturation process. However, the mechanism by which the symptoms are triggered is unexplained.

The neuropathology and the above data are thus supportive of the neurodevelopment hypothesis while familial aggregation, twin and adoption studies are suggestive of a genetic component playing a role as one of the etiological factors contributing to the susceptibility to schizophrenia.

Genetic hypothesis

Schizophrenia is found to occur at an increased rate among biological relatives of patients with schizophrenia as against adoptive relatives. Family, twin and adoption studies indicate that there is a genetic contribution to schizophrenia. However, what is inherited is not the certainty of the disease accompanying a particular genotype but rather a predisposition or liability to develop the disorder.

Twin and Adoption Studies

Twin studies are based on the assumption that monozygotic (MZ) and dizygotic (DZ) twins share a common environment to approximately the same degree. However, MZ twins are genetically identical, whereas DZ twins (like full siblings) share on an average only half of their genes. As both share environmental influences approximately to the same extent, a significantly higher concordance for the disorder in monozygotic (46%) than the dizygotic (14%) twins is indicative of genetic aetiology. Results from twelve major twin studies of schizophrenia agree that proband wise concordance for schizophrenia is much higher in monozygotic twins than in dizygotic twins, though the absolute rate of concordance varies widely which could be due to the broader definition of schizophrenia employed in some studies. Twin studies also provide powerful tests for the role of nongenetic familial transmission in the liability to schizophrenia. All major twin study data suggest that nongenetic factors also play a role in the transmission of schizophrenia.

Adoption studies can clarify the role of genetic and environmental factors in the transmission of schizophrenia by studying two kinds of relationships namely

1. Persons who are genetically related but do not share familial environmental factors and
2. Persons who share familial environmental factors but are not genetically related.

Affected biological parent design: These studies have compared the adopted away offspring of schizophrenic parents with the adopted away offspring of matched controls. Heston found a

significant excess of schizophrenia in adopted away off spring of schizophrenic mothers than in those of control mothers (Heston, 1966). The second study carried out by Rosenthal *et al*, (1968) showed similar results. The third study also indicated a statistically significant excess of schizophrenics in adopted away offsprings of schizophrenic mothers compared with adopted away off spring of matched control mothers.

Affected adoptee design: In this adoption strategy, the ill adoptee rather than the ill parent permits two separate experiments. (i) A test for etiological role of shared environmental factors by comparing the non-biological adoptive relatives of schizophrenic and control adoptee, and (ii) A test for the etiological role of genetic factors by comparing the biological relatives of the schizophrenic and control adoptee who were raised in house holds away from their ill relatives.

Both chronic schizophrenia and schizophrenia spectrum disorder were significantly more common in the biological relatives of schizophrenic proband than in the biological relatives of control adoptee. No concentration of schizophrenia spectrum illness was seen in the adoptive relatives of schizophrenic adoptee. Twin and adoption studies thus provide strong and consistent evidence that genetic factors have a major role in the familial aggregation of schizophrenia.

Slater and Cowie have put forth the single gene hypothesis for schizophrenia (Slater and Cowie, 1971; Elston and Campbell, 1970). However, data from twin and family studies have shown to be incompatible with the hypothesis that, schizophrenia can be accounted for by a mutation in a single gene (McGue and

Gottesman, 1985) or that it represents a collection of single gene disorders each with a similar clinical manifestation (Risch, 1990).

The liability or the threshold model put forth by Falconer (Falconer, 1965) and applied to schizophrenia by Gottesman and Shields (1967) stating that schizophrenia results from the combined action of several different genes, is statistically more satisfactory. This model fits with the observation that the risk for schizophrenia increases with the number of other relatives affected. A model involving several genes is easier to reconcile with the fact that schizophrenia persists at a higher rate in the population despite being associated with reduced reproductive fitness. In accordance to the polygenic hypothesis, what is inherited is not the certainty of the disease accompanying a particular genotype but rather a predisposition or liability to develop the illness. Persons, who are predisposed, develop the illness because environment elicits the disposition of inherited genes, especially if the environment is stressful.

An alternate explanation of the mode of transmission of schizophrenia, which has not been excluded by statistical studies, is the mixed model of inheritance. Here, a gene of major effect acts in combination with a background of polygenes. However, attempts to test this model have yielded inconclusive results (Vogler *et al*, 1991) which could be due to the fact that multilocus models have traditionally assumed that genes contributing to schizophrenia act in an additive fashion. However, family data could show epistatis (Risch, 1990) where two or more loci may have multiple interactions where the final result is more or less than the sum of the effects of individual loci.

MOLECULAR GENETICS OF SCHIZOPHRENIA

Molecular genetics has revealed several mechanisms giving rise to the complex patterns of inheritance and phenotypic variability. These include genomic imprinting and mutations involving unstable DNA sequences in the form of expanded trinucleotide repeat. Imprinting refers to the differential expression of a gene \ set of genes depending on whether it is of maternal or paternal origin (Asherson *et al*, 1994). Expansion of trinucleotide repeats leads to dynamic mutations wherein the change in the size of the repeat number with successive generation results in alteration in disease severity between generations and also irregular patterns of transmission. The two classical approaches that have been used to carry out genetic studies are linkage and association studies.

Location of schizophrenia genes through linkage analysis

Despite the uncertainties about the mode of transmission and implausibility of a single-gene explanation for schizophrenia, most of the efforts have been directed at locating schizophrenia genes by linkage analysis in large families with multiple affected members or by positional cloning where one looks for the segregation of a trait across generations with a marked chromosomal region. Here, sets of polymorphic DNA markers readily identifiable by molecular techniques showing substantial sequence variation among people are used. If a particular region is statistically linked to a trait (affected by illness), that region is then explored for the gene that might be contributing to vulnerability.

Compared with linkage analysis, an independent and a more direct approach for the assessment of susceptibility to schizophrenia by a

specific gene involves the study of specific markers at the candidate gene in ill individuals as compared to matched controls (Mourant *et al*, 1978). If an allele of the marker gene is found more or less frequently in the patient population than the control, then it is said to be 'associated' with the disease. Findings of linkage analysis are the result of genome scans, scans of single chromosome, or chromosomal regions predicted on a candidate gene hypothesis or as follow-ups to earlier studies.

Chromosome 13 linkage findings: Strong linkage findings have been published for chromosome 13. Using 18 polymorphic gene markers in 13 multiple affected families from Europe and Japan, Lin *et al* (1995) have reported linkage to chromosome 13 with Lod score at two. A follow up study in 44 independent pedigrees, however, failed to support linkage. When this replication sample was combined with the original study sample and then divided into groups based on ethnic origin, the Caucasian group gave a positive two point Lod score for several markers. The authors, therefore, speculate that the susceptibility locus for schizophrenia in Caucasians may be different from that in Orientals. Results of genome scan of 54 multiplex families, using 452 micro-satellite markers and covering 22 autosomes with 7.6 centiMorgan resolution, found significant linkage on chromosome 13q32 (Blouin *et al*, 1998). However, a genome wide scan carried out by Shaw *et al* (1998) with 10.5 centiMorgan resolution in 70 multiplex families did not show significant evidence of linkage as per the guidelines of Lander and Keuglyak (1995).

Chromosome 8: Suggestive evidence for linkage to 8p was reported by Pulver *et al,* (1995). A replication effort using 265

multiplex pedigrees supported the existence of a vulnerability locus between D8S1715 and D8S1739 (Kendler *et al*, 1996). Analysis conducted in a new sample of 403 –567 pedigrees using 14 micro-satellite markers yielded suggestive evidence for a linkage to chromosome 8p with Lod score of 2 (Schizophrenia linkage collaborative group for chromosome 3,6, and 8 1996). Two additional reports of evidence for schizophrenia susceptibility loci on chromosome 8 were published (Shaw *et al,* 1998, Kaufmann *et al*, 1998).

Chromosome 22: A number of studies show weak positive linkage on chromosome 22. Initial findings using 38 families gave a Lod score of 2.82 (Pulver *et al*, 1994a). To follow up this suggestive finding a collaborative effort was undertaken. Data from genotyping of 3 dinucleotides repeat polymorphisms in a sample of 256 families provided no evidence of linkage for the region 22 q12-q13 (Pulver *et al*, 1994b). A genome wide scan carried out by Coon *et al*, (1994b) gave positive evidence for linkage on chromosome 22 using both parametric and non-parametric analysis. Later, two studies reported non replication (Kalsi *et al*,1995; Riley *et al*, 1996). A more recent study by Shaw *et al,* (1998) had positive linkage results for chromosome 22. However none of the analysis reported so far meets the significant thresholds established by Lander and Kruglyak, (1995).

A partial genome scan by Wang *et al,* (1995) found suggestive evidence for linkage on chromosome 6pter-p22 in a study in 186 multiplex families with a Lod score of 3.2. Augmentation of the sample by 96 new pedigrees resulted in diminished evidence (Straub *et al*,1995). Another study reported by Moises *et al,* (1995)

found significant linkage for schizophrenia on chromosome 6. A follow up study by Antonarakis *et al*, (1995) investigating susceptibility loci on chromosome 6p24-22 gave a Lod score of 1.17. Schizophrenia Collaborative Linkage Group (1996) found a maximum multipoint sib-pair analysis lod score of 2.2 in a study of 54 families. Recently, NIMH Genetics Initiative Collaborative Study Kaufmann *et al,* (1998) found evidence of linkage, though not significant, on chromosome 6 q16-6q24. Several regions of chromosome 6 have, therefore, been implicated and await further replication.

Chromosome 10: The Genetic Initiative of the NIMH presented results for a European-American sample of 43 pedigrees containing 50 independent sib-pairs. Statistical evidence suggestive of linkage was reported for two markers on chromosome 10p (D10S1423, D10S582) Faroane, (1998). Straub *et al*, (1998) tested a total of 12 markers across 32 centiMorgan region in the region 10p15-p11 of 265 Irish families Multipoint non-parametric analysis peaked at D10S2443. Further evidence for a schizophrenia susceptibility locus in the same 10p region was obtained for the marker D10S1714 (Schwab *et al*, 1998).

Chromosome 5 and others: Sherrington *et al* (1988) reported strong evidence for schizophrenia susceptibility locus on chromosome 5q11-q13. Subsequent studies, however, did not provide support for schizophrenia locus in this region (Crowe *et al*, 1991; Kennedy *et al*, 1988; St Clair *et al*, 1989). More recent studies by Silvermen *et al*, (1996) and Straub *et al*, (1997) however, found evidence for schizophrenia susceptibility locus on chromosome 5.

Additional reports of evidence for schizophrenia susceptibility loci on chromosome 3p Pulver *et al*, (1995), chromosome 4 (Coon, *et al*, 1994b; Shaw *et al*, 1998) chromosome 7 and 14 (Blouin *et al*, 1998) chromosome 20 (Moises *et al*, 1995) and chromosome 9 (Moises *et al*,1995; kaufmann *et al*, 1998) await replication.

As alteration in dopamine transmission has been strongly implicated in the pathogenesis of schizophrenia, Moisis *et al*, carried out linkage analysis using dopamine receptors as candidate genes (1991); Byerley *et al*, (1989) have reported exclusion for the D2 dopamine receptor region. Linkage analysis carried out using all the five-dopamine loci in nine-multigeneration families, which include multiple affected individuals were negative.

Assessment of susceptibility to schizophrenia through association analysis

A direct approach to the assessment of susceptibility to schizophrenia is through association analysis (Mourant *et al*, 1978). Association studies make no assumption about the mode of inheritance and hence are potentially useful for schizophrenia, where the mode of inheritance remains undetermined. Population associations between a disease and a marker can be observed because marker gene itself is a susceptibility gene and the mutant allele detected among patients is presumably inherited from a common 'founder' (Walden and Strom, 1990) or an allele of the marker gene is in linkage disequilibrium with the disease gene. That is disease mutation is co-inherited with an allele of the marker gene because the two loci are physically close together. The distribution of the marker allele in ill individual is, therefore, compared with the same distribution in matched controls. In

association studies, the choice of the marker gene is very important. If an allele of the marker gene is not involved in the pathogenesis of the illness or is not in linkage disequilibrium with the disease allele, an association is unlikely to be found (Cooper and Clayton, 1988).

Association studies in schizophrenia using inherited markers like blood groups, immunoglobulin allotype markers, Gc serum groups, esterase D, acid phosphatase variants as well as polymorphism studies in genes encoding propiomelanocortin, gastrin releasing peptide, stomatostatin, and neuropeptide Y have shown negative results (Lang, 1982; Rinieris *et al*, 1982; Propert, 1983; Feder *et al*, 1985; Rudduck *et al*, 1985; Detera-Wadleigh *et al*, 1988; Fananas *et al* 1990; saha and tsoi, 1990). A suggested association of schizophrenia with the porphobilinogen deaminase gene locus (Su *et al*,1991) is controversial (Nimgaokar *et al*, 1992).

An association study using case control design detected a negative association of schizophrenia with HLA DQB1* 0602 among African-Americans (Nimgaokar *et al*, 1992). Further evidence for the same that is negative association with HLA DQB1* 0602 as well as a positive association with HLA DQB1* 0303 was provided by Nimgaokar *et al* (1995). However, no significant relationship between these alleles and age of onset was noted in these studies.

Though linkage studies using Dopamine receptors were negative, variation at this locus might be associated with more subtle differences in liability to develop schizophrenia. Comparing allele and genotype frequencies between patients and controls for the D3 receptor tested this hypothesis. Two groups of investigators based in Cardiff, UK and Rouffach, France have independently

reported an association of schizophrenia with the dopamine D3 receptor gene (D3RG) locus (Crocq *et al*, 1992). More patients than controls (statistically significant) were homozygous for Bal I polymorphism in the dopamine D3 receptor gene. Homozygosity for the B1 site was associated with only a modest relative risk for schizophrenia. Further studies on relationship between homozygosity at the Dopamine receptor gene and schizophrenia showed an association only in patients with a family history of schizophrenia (Nimgaokar *et al*, 1993) and patients showing a good response to neuroleptic treatment (Mant *et al*, 1994, Jonsson *et al*, 1993).

As alteration in GABAmeric neurotransmission has been indirectly implicated in the pathogenesis of schizophrenia, GABA receptor subunit genes have been studied as plausible candidate genes. Studies carried out by Coon *et al* (1994) indicate a variant found in exon 9 that co segregated in a family with 2 affected siblings and was transmitted to 2 of the 3 affected sibs in another multiplex family. The sequence variant was found at a low frequency in the control group (Coon *et al*, 1994). Genetic association between GABA-A receptor alpha 5 subunit gene locus GABRA (5) locus in 46 schizophrenic Greek patients and 50 controls suggested an association between one allele and a subgroup of patients with late age of onset (over 25 years) (Papadimitrious *et al*, 2001).

The European Multicentre Association Study of Schizophrenia (EMASS) has reported positive association studies on the serotonin receptor in the 5HTR2A gene (Williams *et al*, 1997). However, He *et al*, (1999) report no evidence for association or transmission disequilibrium in 1200 individuals studied at the 102T/ C locus.

Similarly, negative association has been reported by Virgos *et al*, (2001).

As is evident form the data from linkage and association studies, positive findings in one cohort have not necessarily been replicated in further studies. This could be due to several reasons as discussed below.

Factors contributing to the failure of linkage and association studies

There are several reasons why conventional linkage analysis hasn't been successful. In the first instance, most studies involve multiplex families and focus on the detection of the major locus. However, multiplex families do not necessarily indicate the operation of a major gene effect and hence the possibility of more than one susceptibility gene being present in one family cannot be ruled out. Secondly, diagnostic instability that could occur due to late onset in some cases, family members initially classified as unaffected become ill. Finally, incomplete penetrance, phenocopies, heterogeneity and epistatic interactions have to be taken into consideration. As a result the following parameters have to be considered while carrying out segregation analysis.

1. Allowance for incomplete penetrance for a major locus: Considered individuals with high-risk genotype may not express recognizable symptoms of the disorder.
2. Allowance for phenocopies: Affected individuals may not carry any significant predisposing gene but could be affected due to environmental insult.

3. Comparison of models having only one major susceptibility gene or several major genes that interact additively.
4. Inclusion of polygenic components that could interact with one or more major genes.
5. Environmental effects.

Application of association strategy to schizophrenia has included a limited number of candidate gene loci. However, as of today a large number of neurotransmitters, neuroreceptor genes, and genes associated with nuerodevelopment and regulation of the central nervous system are still under study. Association studies can be carried out only in those genes whose sequences are known. A good ethnic and racially matched control population should be available or else false positive association due to population admixes or ethnic differences are a possibility.

NUCLEOTIDE REPEATS IN THE HUMAN GENOME

Repetitive DNA is a major component of all eukaryotic genomes. They are ubiquitous and have been widely exploited in gene mapping, disease gene location and DNA fingerprinting. They can be classified based on their length, copy number, and genomic localization. The most abundant repeat families are of short interspersed elements (SINE Alu) and long interspersed elements (LINE Kpn) which account for roughly 30% of the human genome by mass (Moyziz *et al*, 1989). Less abundant known repeat elements include THE/ Mst 11sequences (Paulson *et al*, 1985), mini-satellites or variable number of tandem repeats (VNTRS) (Jeffrey *et al*, 1985; Nakamura *et al*, 1987), micro-satellites or short tandem repeats (STRs) (Weber and May, 1989) and telomeric and

centromeric alphoid repeats. (Moyziz *et al*, 1987). Mini satellites are several hundred at each locus with a repeat unit of 9-100 bp. They are GC rich, highly polymorphic and extensively used in constructing linkage maps, forensic medicine paternity testing, and population genetics (Jeffrey, 1985; Jeffrey, 1987; Nakamura *et al*, 1988; Hammond, 1994). Micro satellites or short tandem repeats are composed of short motifs (1-6 bp) 5-100 each scattered more or less randomly throughout the genome (Tauz and Renz 1984; Weber, 1990; Beckman and Weber, 1992). The dinucleotide $(AC)_n$ can be found on an average every 30-40 Kb. (Gastrier *et al*, 1995). Majority of the available genetic markers are based on $(AC)_n$ simple sequence repeats. In addition to dinucleotide repeats, tri, tetra and penta repeats are also abundant. However, they have not been comprehensively surveyed to determine their utility as genetic markers. A limited set of genetic markers based on trinucleotide repeats has been published (NI/CEPH Collaborative Mapping Group 1992, Weissenbach *et al*, 1992, Gyapay *et al*, 1994).

Mutation events occurring in the genome

The rate of mutation in the human genome varies depending upon the type of sequences. Unique gene sequences change very slowly at a rate of about 1 amino acid out of 400 per 200, 000 years (Alberts *et al*, 1993). The rate of mutation is static or is the same as that of its predecessor. Repeat sequences associated with the heterochromatin are, on the other hand, in constant state of flux. Their mutation does not usually manifest a gross phenotypic change, due to their remoteness from sequences encoding genes that are more or less confined to the euchromatin. Interspersed repeat DNA sequences or micro-satellites, on the other hand, have

a unique form of mutation. They show a variation in the copy number, different from that of its predecessor and are hence termed as "dynamic mutations" (Richards *et al*, 1992). The expansion of one group of repeats, the trinucleotide or triplet, repeats is known to cause several inherited disorders and this information is given in Table2.

Table 2:Disorders caused due to amplification of trinucleotide repeats

Disease	Repeat	Repeat length	Gene product
Spinal bulbar muscular atrophy	CAG	Normal:11-34 Disease: 40-60	Androgen receptor transcriptional factor
Fragile X syndrome	CGG	Normal: 6-52 Premutation:60-200 Full mutation: 200-2000	FMR –1 RNA binding protein
FRAXE Mental retardation	GCC	Normal: 6-25 Disease > 200	_
Myotonic dystrophy	CTG	Normal: 5-37 Premutation: 50-180 Fullmutation: 200->20000.	Myotonin protein kinase Serinethreonin kinase
Huntington's Disease	CAG	Normal: 11- 34 Disease: 36- 121	Huntington protein
Spinocerebellar Ataxia type 1	CAG	Normal: 7-25 Disease: 41-81	Ataxin -1
Dentatorubral pallidoluysian atrophy	CAG	Normal: 7-25 Disease: 49-88	Atrophin
Machado-Joseph disease	CAG	Normal: 13-36 Disease: 68-79	MIDI gene product

Trinucleotide repeat expansions are unstable mutations that change in size during successive generations. Expansions arise from GC rich triplet repeats that are polymorphic in the normal population, with expansions occurring more often than

contractions. In general it has been observed that longer repeats correlate to more severity of the disease.

IMPLICATIONS OF TRINUCLEOTIDE REPEAT EXPANSION IN SCHIZOPHRENIA

Since classical linkage and association studies are still inconclusive, I decided to explore the role of dynamic mutations in schizophrenia. Their presence may explain the phenomenon of anticipation seen in some families, the higher concordance rates in monozygotic as against dizygotic twins and also the brain and neurodevlopmental hypothesis.

Anticipation: Expanded trinucleotide repeats are unstable and change in size when transmitted to successive generations, with expansions occurring more often than contraction. This property of trinucleotide repeats could explain the phenomenon of anticipation observed in some large families.

Twin studies: Monozygotic twins (46%) show a higher concordance for schizophrenia as compared to dizygotic twins (14%). The discordance observed in dizygotic twins with respect to psychiatric disorders was associated with environmental factors and the interaction between oligomeric genes. The concept of unstable genes provides a very good explanation for the disconcordance observed in monozygotic twins. Assuming each zygote receives a different degree of unstable DNA from the parent germ line; and if the initial level of repeats present in each zygote is much below the threshold level requiring to manifest the disease, each monozygotic twin undergoes cycles of amplification at different degrees and only one of the two may cross the threshold limit. However, if the initial level of expansion in the germ line cells is

very high, very little expansion during the mitotic events could reach the threshold level to manifest the disease. This implies that the disease could be severe with early onset in concordant pairs. Most of the studies with only one exception show a correlation between severity and monozygotic concordance rate. The number of repeats present in an individual beyond a threshold limit would contribute to the difference in outcome observed in individuals suffering from schizophrenia.

Abnormal Neurodevelopment: Abnormal neurodevelopment is well established in schizophrenia (Weinberger *et al*, 1987, 1995). An insult early in development could result in cortical abnormalities seen later in life. Amplified trinucleotide repeats if present within or in the regulatory region of the genes involved in the complex cascade mechanism could affect the regulation of such genes disrupting what is otherwise a highly ordered and regulated cascade. Growth associated protein (GAP), brain derived nerve factor (BDNF), neuronal cell adhesion molecule (NCAD) are some of the candidate molecules likely to be involved in this process (Wickert and Weinberger, 1998).

Amplified trinucleotide repeats have been shown to be the cause of several inherited disorders like fragile X syndrome, myotonic distrophy (DM), Huntington disease (HD), spinocerebeller ataxia (SCA 1), spinal bulbar muscular dystrophy (SBMD), dentatorubal pallidoluysian atrophy (DRPLA) and Machado – Joseph disease (Paulson *et al*,1996).

GENESIS OF THESIS AND OBJECTIVES

It is clear from family, twin and adoption studies that there is an important genetic contribution to schizophrenia. However, the mode of transmission like that of other complex disorders is non-Mendelian in nature. The findings that though the prevalence is little less than 1%, the concordance rate for monozygotic twins is 45% and for the children of 2 schizophrenics is 46% are compatible with a general multifactorial model allowing for polygenic inheritance and environmental effects. Classical genetics, however, fails to explain the mode of inheritance and the phenomenon of anticipation observed in schizophrenia and linkage and association studies are inconclusive.

Hence I decided to explore the role of DYNAMIC MUTATIONS in schizophrenia. The primary objective of this work is to find out if there is any correlation between amplified trinucleotide repeats and the occurrence of schizophrenia. Some candidate genes expressed in the brain were selected for the study. As abnormal neurodevelopment is associated with schizophrenia, polymorphism studies on genes like NOTCH 4 and CAG RI genes that have a critical role in neurodevelopmental aspect, namely cell fate determination, were planed to be carried out. A dysregulation of apoptosis could underlie both of these seemingly divergent processes of neurodevelopment and neurodegeneration and this prompted me to study the involvement of apoptosis in schizophrenia, as the possibility of limited neurodegeneration in concert with a neurodevelopmental disorder cannot be ruled out.

Chapter 2

NUCLEOTIDE POLYMORPHISM IN SCHIZOPHRENIA[1]

[1] The content of this chapter is under preperation as a full length paper

Research strategy used for the study

One of the objectives of my work was to find a correlation between amplified trinucleotide repeats and schizophrenia. The first step in that direction was to screen the database for proteins expressed in the brain having repeats within their coding region. Candidate genes were selected for case control association studies after screening the database for sequences expressed in the brain having more than eight consecutive CAG repeats (see section on selection of candidate genes). As schizophrenia appears to be a multifactorial polygenic disorder showing anticipation, amplified trinucleotide repeats in one or more genes may be involved in the susceptibility to schizophrenia. Hence polymorphism studies were carried out to investigate whether CAG repeat expansions or any allelic variants are involved in the susceptibility to schizophrenia.

Reason to use trinucleotide $(CAG)_n$ in my work

Trinucleotide repeats depending on the repeat class are one or two orders of magnitude less frequent than the $(AC)_n$ repeats (Gastier *et al*, 1995). In the event of a mutation (expansion/regression) occurring with successive generations, the reading frame of the protein is not lost. Presence of such repeats in the 5' upstream or 3' down stream region could affect the regulation of the concerned protein without causing a lethal mutation. So also dynamic mutations occurring within the protein coding region could be tolerated to a certain extent if they are present in the regions not involved in the active site, protein folding or conferring the three dimensional structure. However, expansion of the repeat beyond a threshold value could result in a

pathological condition. Several dominantly inherited neurodegenerative diseases such as fragile X syndrome, spinal and bulbal muscular atrophy, myotonic dystrophy, Huntington disease, spinal cerebellar ataxia type 1, and dentatorubral-pallidoluysian atrophy are caused due to expansion of CAG repeats as detailed in Table 2 (Petronis *et al*, 1996).

A survey of micro-satellites carried out by Raymond Stalling indicates that $(CAG)_n$ is the most abundant micro-satellite found in the gene bank of human DNA sequences (Stalling, 1994). Two other motifs, $(AAT)_n$ and $(TTG)_n$ are nearly as frequent, however, they have not yet been implicated in human genetic disorders. Table 3 shows the distribution of each type of repeat with respect to various categories of genomic sequences, that is, whether the repeat is located in 5' UTR, exon, intron, or 3' UTR. As is apparent from the Table, $(CAG)_n$ repeats are the most frequent trinucleotide repeats found in the exonic DNA sequences (Stalling, 1994). This suggests that $(CAG)_n$ repeats may be more frequently associated with disease syndrome because of their prevalence in exons and that association of this repeat motif with five of the micro-satellite expansion syndrome is not a statistical anomaly.

Table 3: Distribution of trinucleotide repeats with respect to transcribed regions.

Repeat	GGC	GGC_4	CAG	CAG_4	CTG	AAT	TCC	TTC	GAA_4	TTG	CAA	CCA	TGG	ATG	ATC
5'UTR	3	59	1	12	3	2	2	2	0	2	1	0	0	0	1
Exon	5	75	**14**	**96**	4	0	3	0	0	1	2	7	0	2	0
Intron	0	4	0	0	3	11	3	2	2	5	3	1	2	2	1
3'URT	0	2	0	3	1	1	0	0	0	1	0	1	1	0	0

All repeats are n>8, except where noted.

METHODS AND MATERIALS

Search for trinucleotide repeats in human cDNA sequences and selection of candidate genes

All confirmed human cDNA sequences were obtained from ENSEMBL human genome server (http://www.ensembl.org/). The ENSEMBL version 1.0 of May 2001, used in this study, consisted of 22345 human cDNA sequences comprising a total of 28,954,185 nucleotides. The cDNA sequences were searched for occurrence of trinucleotide repeats using the method described in Katti *et al,* (2001). Briefly, a repeat was considered only when the same trinucleotide occurred tandemly for a minimum of 10 times allowing 10% mismatch. A simple sliding window technique was used for the detection of tandem repeats of various classes. $(AGC)_n$ is equivalent to $(GCA)_n$, $(CAG)_n$, $(CTG)_n$, $(TGC)_n$ and $(GCT)_n$ in different reading frames or on a complementary strand. Likewise $(AGG)_n$ is equivalent to $(GGA)_n$, $(GAG)_n$, $(CCT)_n$, $(CTC)_n$ and $(TCC)_n$. Thus 10 classes are possible for trinucleotide repeats. Individual repeat frequencies for each class were determined (Katti *et al*, 2001).

Considering the multifactoral polygenic approach for schizophrenia, it was interesting to investigate whether CAG repeat expansion or allelic variants are involved in increasing the susceptibility to schizophrenia in a few specific genes. The latter were selected by screening the data base for genes expressed in the brain that have more than eight consecutive CAG repeats within the coding regions. This chapter deals with polymorphism studies in CAG repeats in six candidate genes (U-80738, M-34960, M-31776, U-80758, U-80743, U-80741) that are expressed in the brain.

Subjects

Patients with chronic schizophrenia were recruited from Kripamayee Medical Center (Meeraj- Maharashtra state), Sassoon General Hospital and Pune Hospital (Pune - Maharashtra state). Controls matched for sex and age were recruited from the staff and students of National Chemical Laboratory, National Center for Cell Science and above patient recruitment sites.

Demographic characters of patients

75 schizophrenics and 61 controls of Indian origin were used for the present study. All patients satisfied the DSMIV criteria for Schizophrenia. Controls were matched for age and sex. Informed consent was obtained from all the individuals participating in the study.

DNA Analysis

Genomic DNA was extracted from 10.0ml of anticoagulated blood using the proteinase K -chloroform isoamyl alcohol method (Miller *et al*, 1988). To 10.0ml of blood, 10.0 ml of phosphate buffered saline (PBS) and 240µl of NP40 were added. The sample was mixed well and centrifuged at 4000rpm, at RT, for 10 min. The nuclear pellet thus obtained was washed with PBS. At this stage, the nuclear pellet can be stored at –70^{o} C or processed further for DNA isolation. The nuclear pellet was resuspended in 8.0 ml TE (pH 8.0) containing 20µg\ml Proteinase K, 0.5% SDS and incubated at 55^{o} C for 2hr with intermittent shaking. To it 1.5M NaCl (final conc) and chloroform : isoamyl alcohol (equal volume) were added and mixed thoroughly for 40 min. Aqueous phase was separated after centrifugation at 10,000 rpm at RT for10 min. DNA was precipitated by adding 2 volumes of chilled ethanol, washed in

70% ethanol, dried and dissolved in TE. The quality and quantity of DNA were checked by electrophoretic and spectrophotomertric methods.

Primers

Table 4 lists the genes that were selected for the present work. Primers were synthesized for these gene sequences at the primer synthesis facility available in the National Chemical Laboratory, Pune, India and at the Molecular Biology Core facility at the Medical College of Georgia, Augusta, USA. These were later used in PCR experiments to amplify the corresponding loci in DNAs of normal and affected individuals.

Polymerase chain reaction

50ng of genomic DNA was amplified using 50 þM of reverse and forward primer each, 200 mM dCTP, dGTP, dTTP, 25 mM dATP, 1mCi α32P dATP, and 0.8U of Taq DNA polymerase (Perkin Elmer) in a reaction volume of 10.0 ml. The PCR reaction involved initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, annealing between 60°-67°C for 1 min (Table 4), extension at 72°C for 2 min followed by a final extension at 72°C for 5 min. PCR reaction products were electrophoresed on denaturing 6% polyacrylamide gels with 7M urea and visualized by autoradiography. As the difference in allele sizes was multiples of three base pairs, it was assumed that variation in the allele sizes was due to differences in the number of trinucleotide repeat units in the amplified region.

A few primers were used to carry out cold polymerase chain reactions and the details of these are as follows: 100ng of genomic DNA was amplified using 100pmoles of each primer 200 mM dNTP,

1.5 mM MgCl2, 0.8U of Taq DNA polymerase (Perkin-Elmer) in a reaction volume of 20ml. PCR reaction products were electrophoresed on 4% Metaphore agarose gels and visualized by ethidium bromide staining.

Table 4: Genes containing CAG repeats: Primer sequence used and number of alleles obtained

	Gene	Annealing temperature	Primer sequence	Alleles
1	U-80738	67°C	5' ACA CTG GGG TGG AGG GTT GGG 5' CAG GCC TCC CAG GCA TCA CAG	4
2	M-34960	65°C	5' TGG AAG AGC AAC AAA GGC AGC 5' GAG AGT CTG TGA GTG GAA GAG	11
3	M-31776	65°C	5' GAA GCA AAC CCG GAC GCA TCG 5' AGA AGA GCA GGA GCA GGA GCG	2
4	U-80758	67°C	5' TCA CAG GTG GCC AAG GCC CAG 5' GGC AGG TGC AGC GTC AAA GGG	3
5	U-80743	60°C	5' CAT TTC CAG CTT CTC AGG CAG 5' AGC TTT GAT CTG TGC TGG GGA	3
6	U-80741	62°C	5' GCA GTT ACA TCT TCA GCT TTT GC 5' CAG GTG GAA TGG AGA TGA GT	1

RESULTS

Frequency Distribution of trinucleotide repeats in human cDNA sequences

Trinucleotide repeats in human genome cDNA sequences (ENSEMBLE V 1. 0. 0 of May 2001) when searched for trinucleotide repeats (n>=10) detected a total of 660 repeats. As can be seen in Table 5, $(GAG)_n$ and $(CAG)_n$ were the most frequently occurring trinucleotides at n=10 (GAG–169, CAG-157) . When the search was carried out for n= 20, $(CAG)_n$ was found to be the most abundant repeat present on the sequences searched. Analysis of the frequency distribution of repeats within each class revealed that, for the repeat class GAG the most abundant of the trinucleotide was GAG(99) followed by GGA(21), TCC(18), AGG (14), CCT(9), CTC(8) when n=10. This gives a sum total of 169 repeats for the GAG class. Similar analysis for class CAG gave a sum total of 157 with trinucleotide CAG(79) as the most abundant repeat followed by AGC(35), GCA(19), CTG(16), GCT(4) and TGC(4).

When n = 20 the GAG class showed a total of 7 repeats with 6 GAG repeats and 1 GGA repeat whereas class CAG had a total of 23 repeats with 18CAG and 2AGC and 2GCA repeats (Table 6).

Table 5: Frequency distribution of trinucleotide repeats (n>=10) in human genome cDNA sequences classified by class of trinucleotide repeats.

0 AAT	1 ATA	0 TAA	1 ATT	1 TTA	0 TAT	=3
6 AAG	3 AGA	26 GAA	0 CTT	1 TTC	3 TCT	=39
1 AAC	0 ACA	1 CAA	M1 GTT	2 TTG	0 TGT	=5
6 ATG	3 TGA	22 GAT	0 CAT	6 ATC	2 TCA	=39
0 AGT	0 GTA	0 TAG	0 ACT	0 CTA	0 TAC	=0
14 AGG	**21 GGA**	**99 GAG**	**9 CCT**	**8 CTC**	**18 TCC**	**=169**
35 AGC	**19 GCA**	**79 CAG**	**4 GCT**	**16 CTG**	**4 TGC**	**=157**
0 ACG	0 CGA	1 GAC	0 CGT	0 GTC	1 TCG	=2
26 ACC	11 CCA	22 CAC	6 GGT	3 GTG	0 TGG	=68
22 GGC	26 GCG	14 CGG	18 GCC	16 CCG	3 CGC	=99
46 AAA	33 TTT	0 GGG	0 CCC			=79

Total repeats (n >= 10) = 660.

Table 6: Frequency distribution of trinucleotide repeats (n>= 20) in human genome cDNA sequences classified by class of trinucleotide repeats.

0 AAT	0 ATA	0 TAA	0 ATT	0 TTA	0 TAT	= 0
0 AAG	0 AGA	7 GAA	0 CTT	0 TTC	0 TCT	= 7
0 AAC	0 ACA	0 CAA	0 GTT	0 TTG	0 TGT	= 0
0 ATG	0 TGA	4 GAT	0 CAT	1 ATC	0 TCA	= 5
0 AGT	0 GTA	0 TAG	0 ACT	0 CTA	0 TAC	= 0
0 AGG	**1 GGA**	**6 GAG**	**0 CCT**	**0 CTC**	**0 TCC**	**= 7**
3 AGC	**2 GCA**	**18 CAG**	**0 GCT**	**0 CTG**	**0 TGC**	**= 23**
0 ACG	0 CGA	0 GAC	0 CGT	0 GTC	0 TCG	= 0
6 ACC	2 CCA	0 CAC	0 GGT	2 GTG	0 TGG	= 10
1 GGC	0 GCG	0 CGG	0 GCC	0 CCG	0 CGC	= 1
0 AAA	0 TTT	0 GGG	0 CCC			= 0

Total repeats (n >= 20)= 53

Thus with the increase in n value from 10 to 20, CAG repeat was found to be present in a significantly higher number as against GAG repeat. Since amplified $(CAG)_n$ repeats are also found to be associated with some neurodegenerative diseases, I decided to explore the possibility of amplified $(CAG)_n$ repeats being involved in the susceptibility to schizophrenia.

Selection criterion for candidate genes

The shortest observed allele size associated with highly polymorphic trinucleotide repeat based STSs was found to be eleven for AAC, seven for AAG, fourteen for AAT, nine for ACC, nineteen for ACT, eight for AGC, seven for AGG and seventeen for ATC indicating that repeats less then seven or eight units are stable (Stalling *et al*, 1994). Variation in mutation rate is likely to occur at longer threshold lengths. Longer repeats being unstable have implications in genome organization, genetic variation and disorders on a much shorter time scale. Hence the minimum repeat unit length for the selection of candidate genes was taken as eight.

Allelic distribution of (CAG)n repeats in the selected six genes

Using the primer pairs as mentioned in Table 4, polymorphism studies were carried out with affected individuals and controls. All the six genes showed alleles in the range of 1-11 and revealed polymorphism except one gene (Table 4). Gene U80741 showed a monomorphic pattern and hence was not considered informative in the present study. The alleles found in M-31776 differed in size by 30 repeats, with allele sizes of 130bp and 180bp as seen in Fig 1 and Tables 7 and 8.

Figure 1: Allele frequency distribution in patients and controls for the gene M- 31776

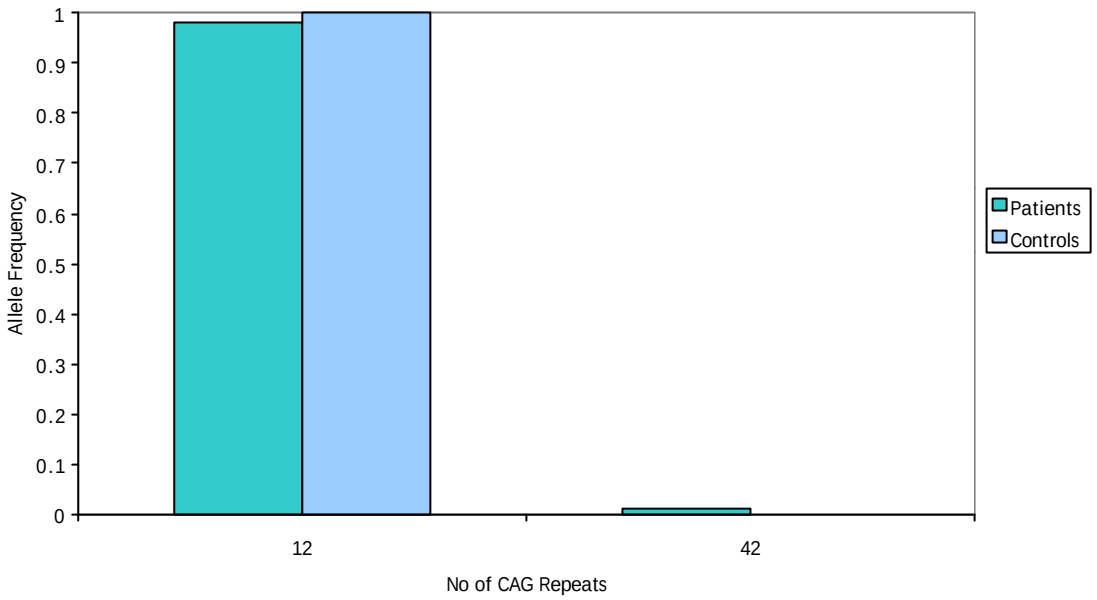


Figure 2: Allele frequency distribution in patients and controls for the gene U-80743

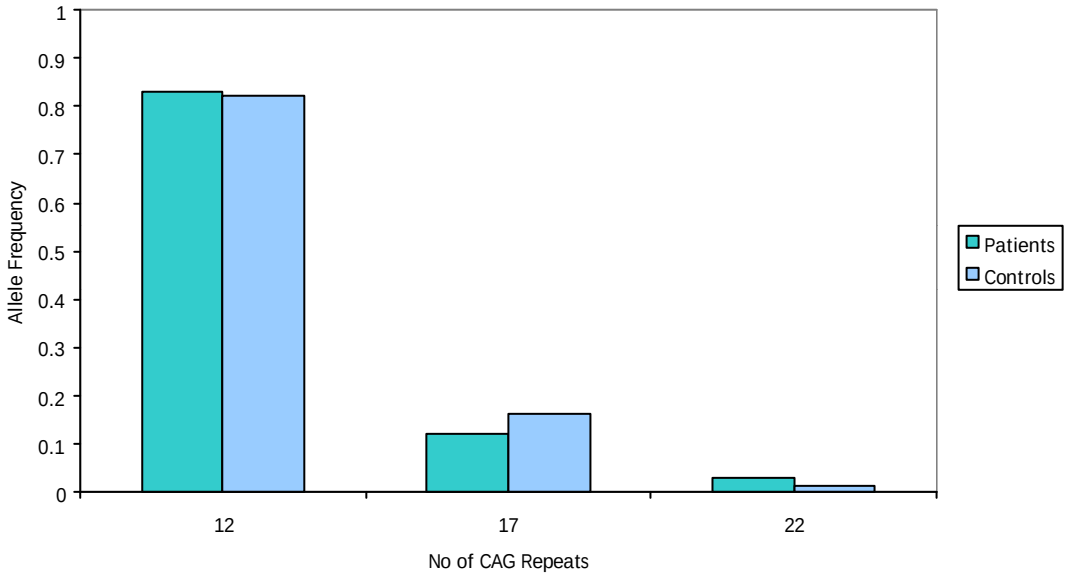


Table 7:Number of alleles and their frequency distribution for the gene M-31776

No.	Allele size (bp)	Repeats	Frequency in patients	Frequency in controls
1.	130	12	0.98	0.01
2.	180	42	1.0	0.0

Table 8:Distribution of allelic combination observed for the geneM-31776

No.	Allele pair	Frequency in patients	Frequency In controls
1.	(130, 130)	0.98	1.0
2.	(130, 180)	0.01	0.0

Table 9:Number of alleles and their frequency distribution for the gene U-80743

No.	Allele size (bp)	Repeats	Frequency in patients	Frequency in controls
1.	170	12	0.83	0.82
2.	185	17	0.12	0.16
3.	200	22	0.03	0.014

The larger size allele was observed in only one affected individual and hence this data was not statistically relevant. Gene U-80743 was found to have amplified three alleles with sizes of 170bp, 185bp and 200bp representing 12,17 and 22 repeats respectively having allelic combinations of (200,170), (185,170), and (170,170) as can be seen in Fig 2 and Tables 9 and 10. Similar was the case with gene U-80758, where three different allelic sizes namely 151bp, 166bp and 187bp representing 21, 26 and 32 repeat units respectively, gave allelic pairs of (187,166), (166,166) and (151,151) (Fig 4). As is apparent from Table 9,10,11and 12 the allele frequency distribution between the patients and controls was not statistically significant.

In case of M-34960 which is a transcription factor IID gene, eleven alleles were present with the number of repeat units spanning between 28 to 39 representing alleles with sizes between204 bp to 237bp as shown in Fig 3 and listed in Table 13. The allele with repeat units of 35 was the most frequent allele in the population. Fig 5 is a representative autoradiogram obtained after denaturing PAGE of amplified PCR fragments for the gene M-34960 in a representative sample population. Lane M on both the ends of the autoradiogram represents the end-labeled phage X-174 Hae III digest.

Table 10: Distribution of allelic combination observed for the gene U-80743

No.	Allele pair	Frequency in patients	Frequency In controls
1.	(200, 170)	0.07	0.02
2.	(185, 170)	0.25	0.32
3.	(170, 170)	0.66	0.64

Table 11: Number of alleles and their frequency distribution for the gene U-80758

No.	Allele size (bp)	Repeats	Frequency in patients	Frequency in controls
1.	187	32	0.22	0.02
2.	166	26	0.07	0.18
3.	151	21	0.70	0.60

Table 12: Distribution of allelic combination observed for the gene U-80758

No.	Allele pair	Frequency in patients	Frequency In controls
1.	(187-166)	0.45	0.40
2.	(166, 166)	0.07	0.18
3.	(151, 151)	0.47	0.40

Figure 3: Allele frequency distribution in patients and controls for the gene M-34960

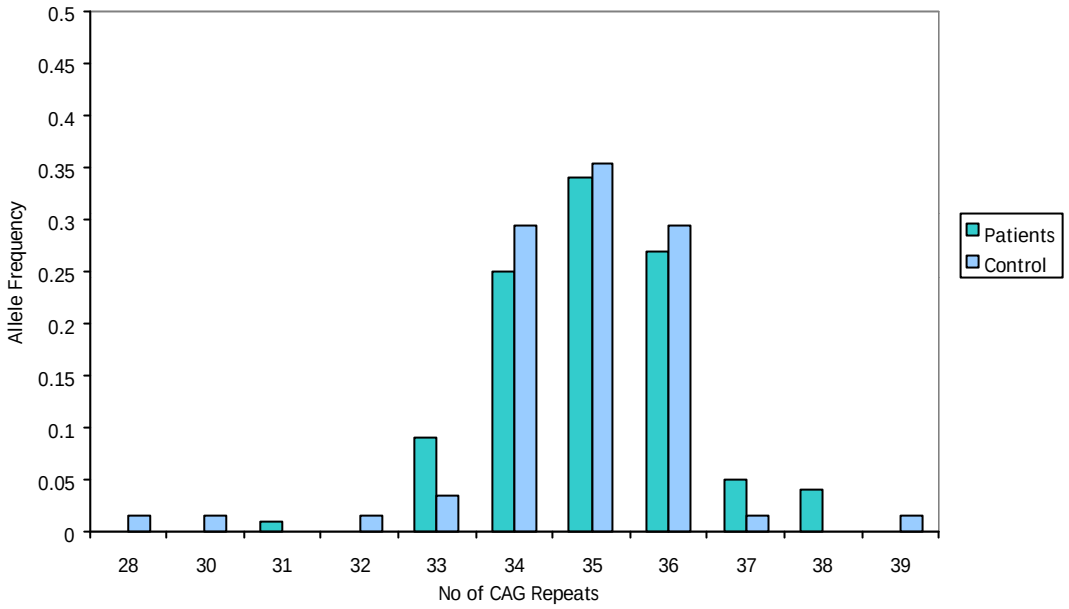
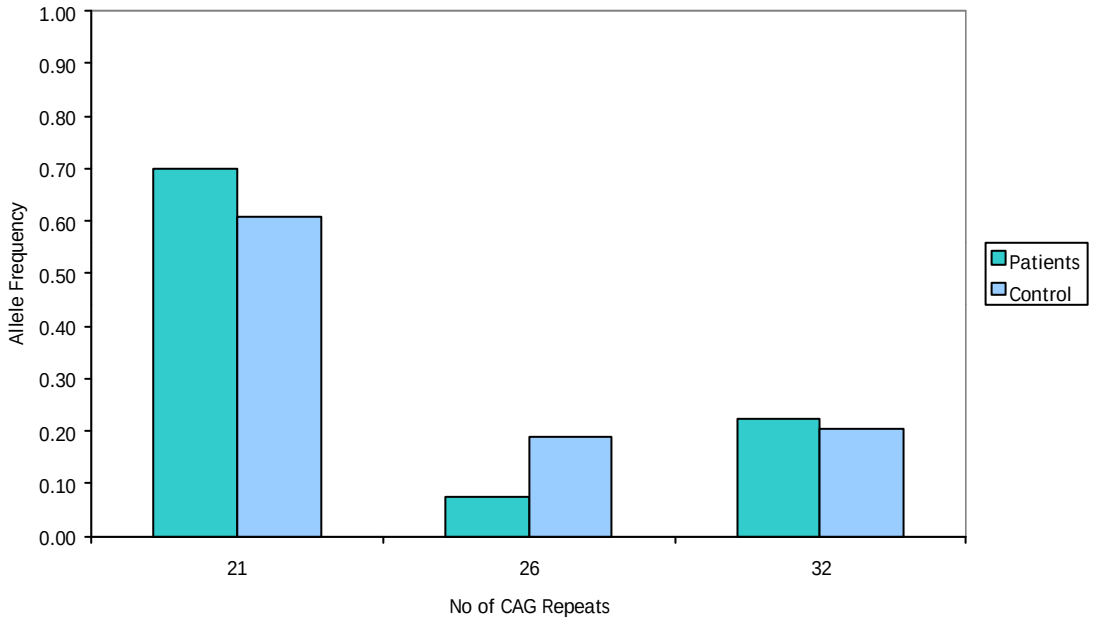


Figure 4: Allele frequency distribution in patients and controls for the gene U-80758



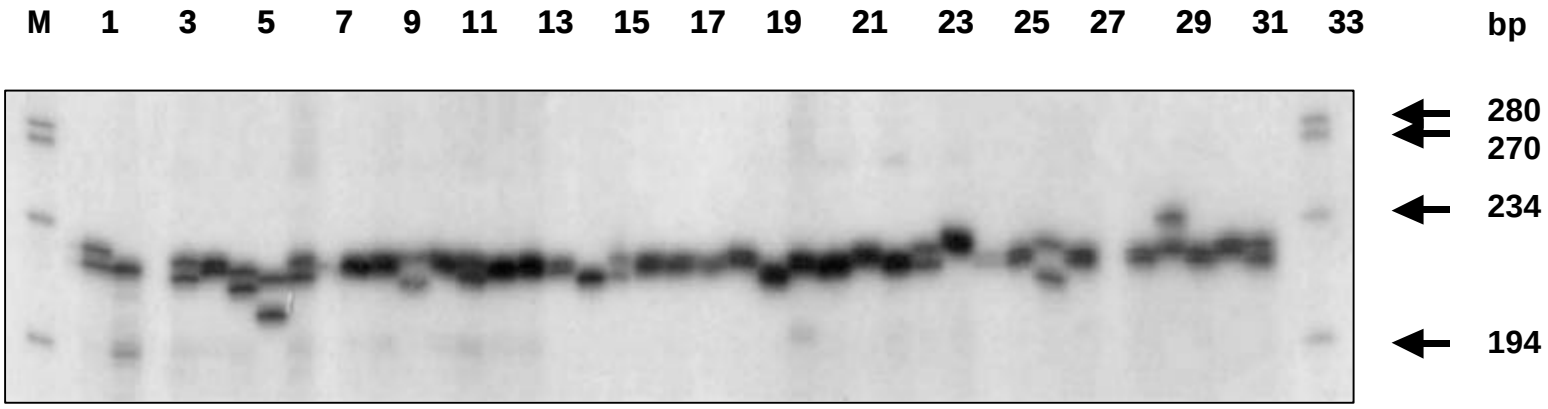
Tables 14,15 and Fig 6 represent the number of alleles and the frequency distribution of alleles for the gene U-80738. Amplified products represented Allele sizes of 149bp, 152bp, 167bp, and 173bp representing repeat units of 15, 16, 21, and 23 respectively. Three allele pairs namely heterozygous (173,152) combination, heterozygous (167,149) combination and a homozygous (149,149) combination were found in the population studied. The allele pair with repeat units of 21 and 15 (167,149) accounted for most of the size difference between affected individuals and controls. The heterozygous condition for this allele pair was found to be significantly higher in controls when compared to affected individuals (α^2= 9.4 and p =0.001). When a sequence homology search was performed using the U80738 nucleic acid sequence against the nonredundant database, U80738 was found to be homologous to nuclear matrix transcription factor.

Figure 7a (patients) and 7b (controls) are representative photographs for amplified PCR products for U-80738 resolved on 3.5% metaphor agarose gel. Lane no 1 in Fig 7b and lane no 24 in Fig 7a represents phage X-174 Hae III digest marker.

Table 13: Number of alleles and their frequency distribution for the gene M-34960

No.	Allele size (bp)	Repeats	Frequency in patients	Frequency in controls
1.	204	28	0. 0	0. 02
2.	210	30	0. 0	0. 02
3.	213	30	0. 01	0. 0
4.	216	32	0. 0	0. 02
5.	219	33	0. 09	0. 04
6.	222	34	0. 25	0. 03
7.	225	35	0. 34	0. 36
8.	228	36	0. 27	0. 03
9.	231	37	0. 05	0. 02
10.	234	38	0. 04	0. 00
11.	237	39	0. 0	0. 02

Figure 5: Autoradiogram of Amplified PCR products for gene M-34960



Lanes 1-40: PCR amplified products in the population studied and lanes M: end-labelled ϕX-174/Hae III digest MWM (bp) resolved on a 6% denaturing page gel

Figure 6: Allele frequency distribution in patients and controls for the gene U-80738

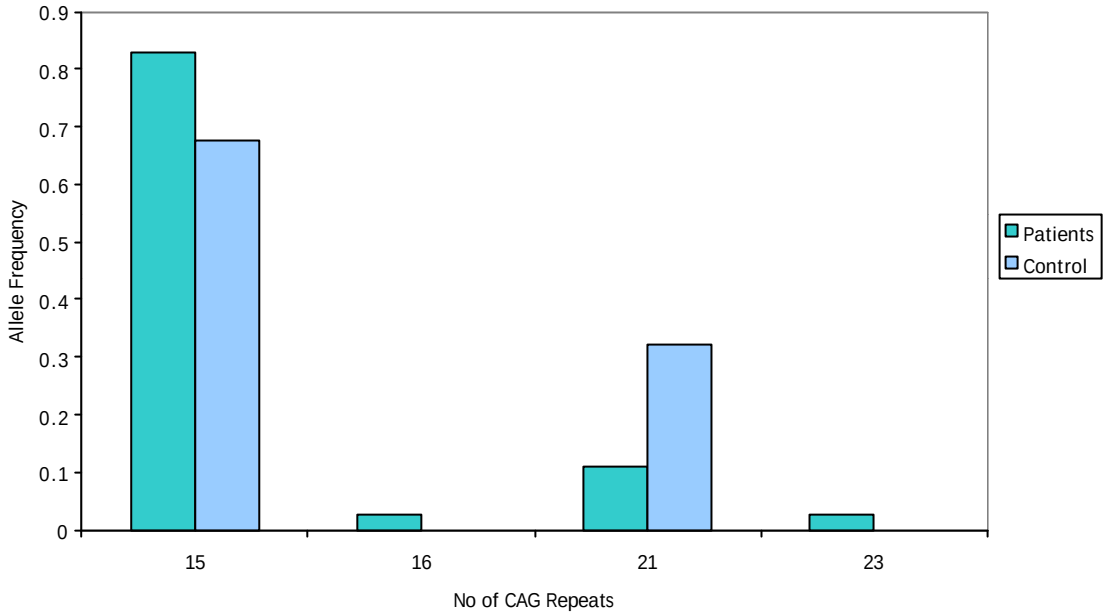


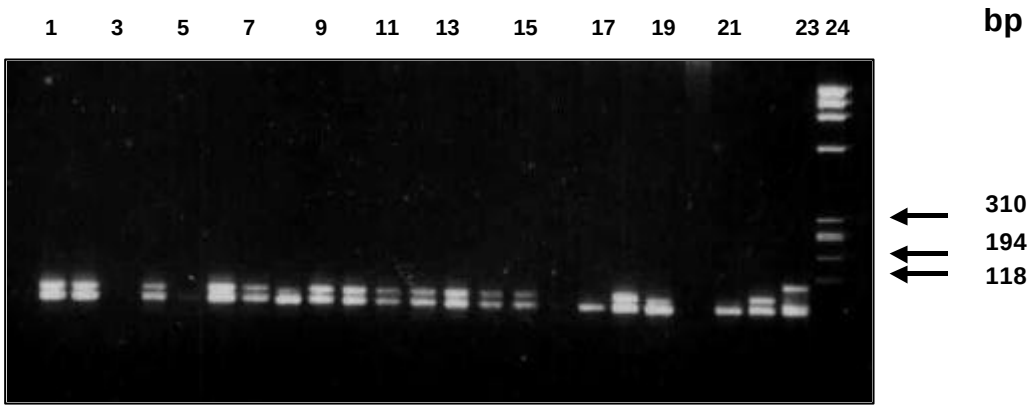
Table 14: Number of alleles and their frequency distribution for the gene U-80738

No.	Allele size (bp)	Repeats	Frequency in patients	Frequency in controls
1.	149	15	0.83	0.67
2.	167	21	0.11	0.32
3.	152	16	0.02	0.0
4.	173	23	0.02	0.0

Table 15: Distribution of allelic combination observed for the gene U-80738

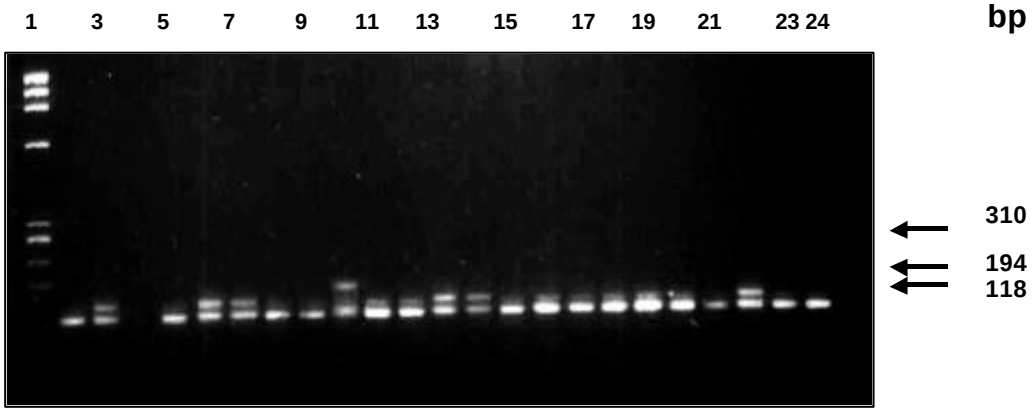
No.	Allele pair	Frequency in patients	Frequency In controls
1	173:152	0.05	0.0
2	167:149	0.22	0.64
3	149:149	0.72	0.35

Figure 7a: Amplified PCR products for gene U-80738



Lanes 1-23: PCR amplified products from controls;
Lane 24: End labelled ϕX-174/Hae III digest MWM (bp)
resolved on 3.5% metaphor agarose gel.

Figure 7b: Amplified PCR products for gene U-80738



Lanes 1-23: PCR amplified products from patients;
Lane 24: End labelled ϕX-174/Hae III digest MWM (bp)
resolved on 3.5% metaphor agarose gel.

DISCUSSION

Several groups have used the repeat expansion detection method (RED) of Schalling *et al,* (1993) to look for evidence of large CAG/CTG repeats in schizophrenic individuals versus controls. For example, O'Donovan et al, (1995) and Morris *et al,* (1995) have shown evidence of expanded CAG/CTG repeats for the same. The locus / loci associated with the disease have not been identified so far as RED is a multi locus assay. However family and monozygotic twin based study carried out by Vincent *et al,* (1998) and parent offspring pairs of patients affected with schizophrenia showing anticipation (Martorell *et al,* 1999) have failed to detect any evidence for large repeats. Similarly, findings of Ohara *et al,* (1997a, 1997b) and Petronis *et al,* (1996) do not support the hypothesis that large CAG/CTG repeat expansions are the major etiological factors in schizophrenia. RED reaction has shown the presence of CAG repeats that are highly variable in size within the control population and across ethnic groups (Sirugo *et al*, 1997). It is possible that moderately expanded repeats are more likely to be associated with schizophrenia. The high number of small expansions found in the control population would limit the ability of RED technique to detect moderate expansions that may be associated with schizophrenia.

In the six genes used in the current study, no association between large trinucleotide repeats (the size found in neurodegenerative diseases) and schizophrenia was found. Reports on RED reaction are also indicative of moderate rather than large repeats being involved with schizophrenia. Among the six genes analyzed in this

study, gene U-80738, with homology to the nuclear matrix transcription factor, showed significant allele frequency variation in patients compared to controls. Heterozygous condition for allele pair having repeat size 15 and 21 was over represented in control (Tables 14 and 15).

The aetiology of schizophrenia appears to be multifactoral, involving a combination of environmental and inherited genetic risk factors. Linkage studies suggest that there are several loci involved in the disease (Moldin and Gottesman, 1997) and is likely to be associated with low penetrance but common functional variations in a number of susceptibility genes (Gottesman and Shield, 1967). It also exhibits anticipation that is explained by trinucleotide repeat expansion. The repeat if present in the 5' noncoding region can affect the expression of the gene. The spatial and temporal expression of the genes coupled with environmental effects can bring about subtle changes affecting the vulnerability of the individual to schizophrenia. Hence our approach was to screen (CAG) repeat containing genes expressed in the brain. Though no association of schizophrenia was observed with CAG repeats, the possibility of moderate to small repeats being involved and also a particular allele pair being found more often in patients than the controls cannot be ruled out.

The alternate approach of screening candidate genes for association in case control studies is labour intensive and each gene has a low prior probability of being involved. Potassium channel gene hsKCa3, and 5HTR2A gene represent two examples of such genes. An association was observed between the Potassium channel gene hsKCa3, which contained two polyglutamine arrays.

A significant difference in the allele frequencies was obtained between schizophrenic patients and the control population with an excess of longer allele in patients. Repeats more than 19 CAG were found to have an increased frequency in schizophrenic individuals (Chandy *et al*, 1998). However, subsequent reports did not always replicate this finding and differences in different populations were apparent. Serotonin receptor 5HRT2A gene polymorphism was reported to be associated with schizophrenia (Williams *et al*, 1997). Schizophrenics with A2A2 genotype were characterized by significantly higher values for negative symptoms (Golimbeqt, 2000). However, several other groups were not able to replicate the positive findings (Verga *et al*, 1997, Virgos *et al*, 2001). Work carried out earlier by several researchers (Speight *et al*, 1997; Jain *et al*, 1996; Sasaki *et al,* 1996) has excluded the role of CAG repeat expansion in the candidate genes studied by them. Joober *et al,* (1999) analysed 14 CAG repeat-containing genes for putative association between allelic variants or expansion of CAG repeat containing genes and schizophrenia and it's variability with respect to responsiveness to conventional neuroleptics. No CAG repeats in the range of those observed in neurodegenerative diseases were identified in 14 genes studied by them (Joober *et al,* 1999). Comparison among the different groups indicated that neuroleptic responders have shorter alleles when compared to controls in one of the genes studied, implicating the possible contribution of this gene (hGT1) to the risk for schizophrenia or in modifying the disease phenotype with regard to outcome and\or neuroleptic responsiveness.

It is apparent from the above discussion that association studies for most of the genes studied so far in schizophrenia are not

consistent. Our data indicates heterozygous condition in controls for the gene U-80738 to be significantly higher as against affected individuals. As indicated by homology search, gene U-80738 is homologous to the nuclear matrix transcription factor and this could represent an example of heterozygote advantage (Cavalin-Sforza and Bodmen 1971). Two different forms at the nuclear matrix transcription factor level could be better suited in bringing about a change at the regulatory level in one or more genes involved in early neurodevelopment and in the increased ability to respond adaptively to variations in environment. As more than one gene is likely to be involved with a low penetrance, multiple testing with respect to various parameters could prove to be beneficial in the long run. For instance, subgrouping patients as neuroleptic responders or non responders, early onset or late onset, and segregation on the basis of sex or stress levels could prove to be informative.

In case- control studies, population stratification is a major factor. Population based case control studies have several limitations. Hence the possibility of association due to population stratification cannot be ruled out and may be responsible for the difference observed between patients and controls in the present study. It is, therefore, necessary to replicate these results in a larger sample preferably with parent offspring trios to confirm the possibility of the heterozygous allele pair predominance in control individuals.

Chapter 3

ROLE OF NEURODEVEOPMENTAL GENES IN SCHIZOPHRENIA

INTRODUCTION

The efficient functioning of the brain requires specific interconnections between nerve cells The nervous system develops in a series of programmed steps that are precisely timed with a temporal sequence that is characteristic of each brain area. As a result of this each neuron connects with target cells only and not any other cells.

The total number of interconnections between nerve cells is approximately 10^{15}. However, the total genetic information available to the animal is approximately 10^5 genes. This is not sufficient to specify the 10^{15} inter connections between nerve cells. This implies that the development of the nervous system must also involve epigenetic processes that sequentially activate and modulate relevant gene pools within the developing cells (Principles of Neural Science, Ed Kandel, and Schwartz, Elsevier. NewYork. Amsterdam. Oxford). Epigenetic influences which impinge on the developing cell arise prenatally from embryonic and postnatally from the external environment. They include nutrition, sensory and social experiences, and learning.

The neurodevelopment hypothesis for schizophrenia

As described earlier in chapter 1 the development of nervous system involves a cascade of events. A fault in any one of the genes involved directly or indirectly in these events may affect brain development and may lead to susceptibility to schizophrenia in two general ways.

1. It can be speculated that a gene normally transcribes a protein that is important for the normal brain

development, and that the wild type allele of this gene becomes activated only at the time of early adulthood when the protein product becomes necessary.

2. Defective genes may impart susceptibility to the illness by sensitizing the organism to environmental adversity. It has been postulated that the 'lesion' results from an abnormal prenatal, or early postnatal adverse environment that affects the developing brain and alters the normal course of developmental events. (Weinberger, 1995).

   The brain lesion in schizophrenia could be caused by defective gene(s) or altered expression of a normal gene(s) due to environmental factor(s) or more likely a combination of both. A diffuse and heterogeneous histopathology, found in the limbic system, diencephalon, and prefrontal cortex probably occurs in early development long before the psychopathology. These data are more consistent with a neurodevelopmental model in which a fixed lesion in early life may influence the normal brain maturation events that occur much later.

Development of the nervous system

Initially, brain development is driven by a genetic program that activates a molecular cascade of events leading to turning on and off particular genes in the cell (Gilbert, 1991; Jacobson, 1992; Ptashne, 1992). It is critical that correct spatial and temporal profile of gene expression is maintained for cells to differentiate producing specific lineages for the normal development of the brain (Rubeinstein *et al*, 1994). Further growth and specification

of cell fate relies increasingly on molecular signals extrinsic to the developing brain. Thus, the coordinated brain development resulting from activation and inactivation of genes in specific cells produces a spatial and temporally unique pattern of proteins that, in turn, determine the capability of cells for sending or receiving messages to neighbouring cells.

The development of different types of neurons and the precision with which they interconnect is programmed. The properties and the function of each neuron in the brain is determined not only by the chemical messengers that it releases, but also by the chemical input it receives from its immediate surroundings The development of the nervous system proceeds in the following manner:

1. Initial step in development: 'determination' ensures that a certain cell population will give rise to the cells of the nervous system. Distinct genes are involved in the initial rostro-caudal cell positioning that regulates the orientation of parallel segments of the anterior and posterior body axis.
2. Cell differentiation wherein cells descending from the determined cell pool give rise to specific population of neurons characteristic of different regions of the nervous system. These neurons in turn proliferate and migrate to the appropriate locations and subsequently make highly specific connections with the targets.
3. Finally, synaptic connections are established to generate appropriate behaviour.

Stable synaptic connections result from a sequence of events, broadly divided into two sequential stages. First is the matching stage where there is selective recognition of the proper target cells by the out going axons of the presynaptic neurons. This is followed by the sorting and adjustment stage, in which, interactions between nearby afferent terminals refine the map and increase its precision.

The outgoing axons selectively recognise the target cells although some fine details of the interactions between neurons, and neuron and muscle, are regulated by environmental factors. The recognition of one cell by another cell is quite specific, requiring pre-existing information on the part of both pre and post synaptic cells though the exact mechanism is not known. The final step in synapse formation and stabilization involves two major interactions between the out growing nerve fiber and the target as follows:

1. The matching of the size of the population of presynaptic neurons and their targets is achieved by cell death, synapse retraction, and competition for growth substance.
2. Fine tuning of connections by competition and activity.

Although the initial establishment of connections occurs in the absence of learning, it is important for subsequent fine-tuning and maintenance as well as for regulating the strength of the connections.

The research strategy used was similar to that in chapter 2. Here however genes critical from the neurodevelopment aspect having

a stretch of more then eight CAG repeats were used. This chapter deals with polymorphism studies in two genes namely the NOTCH4 gene and CAGR1 gene a human homologue of C. elegans cell fate determining gene mab-21.

NOTCH 4 as a candidate gene for schizophrenia

The NOTCh 4 gene is located at 6p 21.3, a site which several studies have shown significant linkage with schizophrenia. Notch signalling is an evolutionarily conserved mechanism that controls an extraordinarily broad spectrum of cell fate and developmental process in organisms ranging from sea urchins to humans (Artavanis - Tsakonas *et al*, 1995). Notch is involved in both lateral and inductive signaling. The mechanism of lateral signaling allows an individual cell or a group of cells to be singled out from the surrounding equivalent cells. The second type of regulatory signalling namely inductive signalling operates between non-equivalent cells. Hence, the signalling cell and the receiving cell begin the process with different properties including their repertories of cell- surface receptors and ligands. Induction of R7 cell fate by the R8 cell during Drosophila eye development is an example of inductive signaling (Aartavanis – Tsakonas *et al*, 1999) In Drosophila, embryos without a zygotic supply of functional NOTCH protein, virtually all cells in the ventral ectoderm adopt the neuroblast fate, which leads to a lethal hypertrophy of the nervous system or adopt the neurogenic fate.

Signals transmitted through the NOTCH receptor, in combination with other cellular factors, influence differentiation, proliferation, and apoptotic events at all stages of development. NOTCH gene therefore, appears to function as a general developmental tool

that is used to direct cell fate and consequently to build an organism and hence can be taken as a candidate gene for the neurodevelopment hypothesis.

CAG R1 as a candidate gene for schizophrenia

CAGR1 gene has been mapped to chromosome 13q13 and its sequence contains a long open reading frame encoding a 359 amino acid polypeptide. This sequence is 56% identical to the *C. elegans* mab-21 deduced amino acid sequence, with 81% amino acid conservation. mab-21 was first defined as a mutation resulting in abnormal development of one set of the nine pairs of peripheral sense organs, known as rays, found in the posterior of male *C. elegans*. These abnormalities in the ray structure of the tail, and abnormalities of morphology, movement and reproduction, suggest that mab-21acts to specify cell fate (). Expression of CAG R1 is higher in brain than in other tissues and within the brain it is the highest in the cerebellum (Margolis *et al* 1996). An anomaly at the expression level of CAGR1can affect the development of the brain.

METHODS AND MATERIALS

Subjects

Patients and controls used for this analysis were recruited from the sources as listed in chapter 2. Fifty- three patients and 50 controls were used for this study.

DNA Analysis

Genomic DNA was extracted by the method as described in chapter 2.

Primers

Primers were synthesised at the primer synthesis facility of the National Chemical Laboratory, Pune, India using the phosphoramidite chemistry.

Primers for the gene NOTCH4

Forward primer: 5'GGAGCAGAGGAGGTGGCTCCTG 3'

Reverse primer: 5'GGCAGTGAGAATCTCCTCCATCC 3'

Primers for the gene CAGR1

Forward primer: 5'GATAAAAGGAAGGGAAAA 3'

Reverse primer: 5'CAGAAATGGATCAAAAAT 3'

Polymerase chain reaction for NOTCH4

50 ng of template DNA was amplified using 50 ρM of forward and reverse primer each, 200 mM dCTP, dGTP, and dTTP, 25 mM dATP, 1mCi α32 P dATP and o.8U Taq DNA polymerase (Perkin Elmer) in a reaction volume of 10 ml. The PCR reaction involved initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, annealing at 70°C for 1 min, and extension at 72°C with a final extension at 72°C for 5 min. The PCR reaction products were electrophoresed on denaturing 6% polyacrylamide gel with 7M urea and visualized by autoradiography.

Polymerase chain reaction for CAGR1

50 ng of template DNA was amplified using 50 μM of forward and reverse primer each, 200 mM dCTP, dGTP, and dTTP, 25 mM dATP, 1 mCi α32 P dATP, o.8U Taq DNA polymerase (Perkin Elmer) and

0.15ml formamide in a reaction volume of 10 ml. The PCR reaction involved initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C with a final extension at 72°C for 5 min. The PCR reaction products were electrophoresed on denaturing 6% polyacrylamide gels with 7M urea and visualized by autoradiography

RESULTS AND DISCUSSION

The possibility that schizophrenia is a neurodevelopmental disorder poses a challenge to neurodevelopmental scientists and neurobiologists in two broad areas. One is the identification of a molecular lesion in the schizophrenic patients and second is the molecular trigger of the phenotypic dysfunction. With the advent of molecular techniques, several developmentally important molecules have already been discovered. It is important to search for aberrant expression or function of developmentally important molecules that are capable of interacting with brain elements over a long period of time. It is unlikely, however, that any early acting gene products are altered in schizophrenia, as this might lead to a much greater alteration in gross brain morphology than the subtle ones found in schizophrenic brain. It is likely that the altered regulation of the product of such genes would be instrumental in triggering or bringing about the change leading to the predisposition rather than the gene product itself being altered.

Analysis of the NOTCH 4 locus

To study the involvement of the NOTCH 4 gene as a risk factor for schizophrenia, polymorphism studies at the (CTG) repeat locus in

the exon1 of the NOTCH 4 gene were carried out. The NOTCH 4 gene revealed 5 alleles in the population studied and Figure 8 is a representative autoradiograph of the NOTCH4 gene analysis. As can be seen in the Fig 8, lane no 7 represents the marker DNA f X Hae III; lanes 1 - 6 and 8 -16 represent PCR amplified products resolved on 6% denaturing PAGE gel.

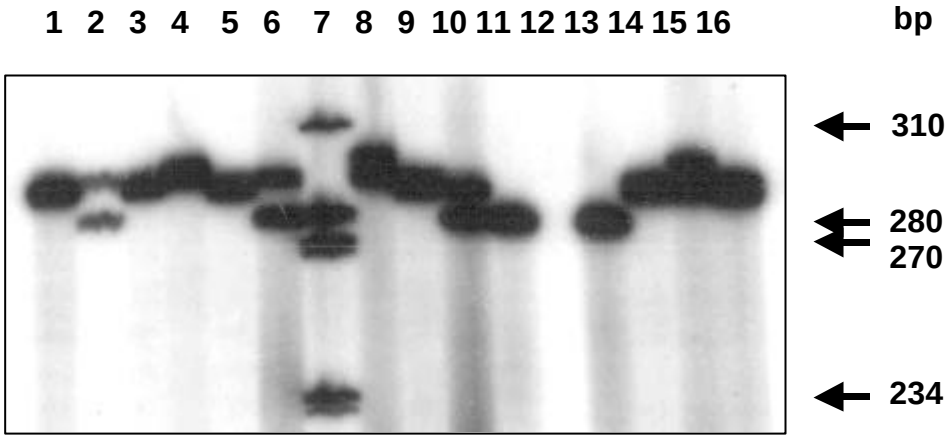
Table 16 shows the allele frequency obtained in patients and controls. Statistical analysis of the data revealed no association between the locus studied and schizophrenia. The chi-square value was found to be 13.7 and the level of significance was p = 0.5. No significant difference in the genotype or allele frequencies of repeat numbers was found between patients and controls.

Table 16: Allele frequencies of $(CTG)_n$ repeats for NOTCH4 gene in patients and controls.

Allele size (bp)	Frequency in patients	Frequency in controls
281	0.05	0.03
284	0.15	0.17
288	0.50	0.58
294	0.10	0.05
302	0.17	0.14

Chi- square $\chi 2 = 13.7$, $p = 0.5$.

Figure 8: Autoradiogram of amplified PCR products for NOTCH-4 gene



Lanes 1-6 and 8-16: PCR amplified products in the population studied;
Lane 7: End labelled ϕX-174/Hae III digest MWM (bp)
resolved on 6% denaturing PAGE gel.

NOTCH 4 is located at 6p21.3 a site that several studies have shown to have a linkage with schizophrenia (Kendler *et al*, 2000; Nurnberger *et al*, 1999; Schwab *et al*, 1995; Straub *et al*, 1995). Wei and Hemmings analysed 13 loci spanning about 1.8 Mb in the MCH region located at 6p21.3 and found several loci showing significant association with British schizophrenic patients (Wei and Hemmings, 2000). Among these, the locus that showed the most significant association p value, p= 0. 000036 were for a (CTG) repeat present in the exon 1 of the NOTCH 4 gene.

To confirm the findings of Wei and Hemmings (2000), regarding the involvement of the NOTCH4 gene as a susceptibility gene for schizophrenia, it was essential to replicate the study in other racial and ethnic groups. A case control study carried out in the Japanese population did not show significant association with schizophrenia or schizoaffective disorders. Three loci in the NOTCH4 region positive in the original study, SNP1 in the 5' flanking region, SNP2 in the promoter region and the CTG repeat in the exon1 of the NOTCH4 gene were genotyped (Ujike *et al*, 2001). Another independent report of $(CTG)_n$ repeat polymorphism in the NOTCh4 gene suggested a lack of association between NOTCH4 gene triplet repeat and schizophrenia in Japanese individuals. The British study (Wei and Hemmings, 2000) employed the transmission disequilibrum and haplotype based tests using parent offspring trios. Both the Japanese groups used case control association study that is less sensitive. A large population based case control study of 300 Scottish schizophrenics and controls

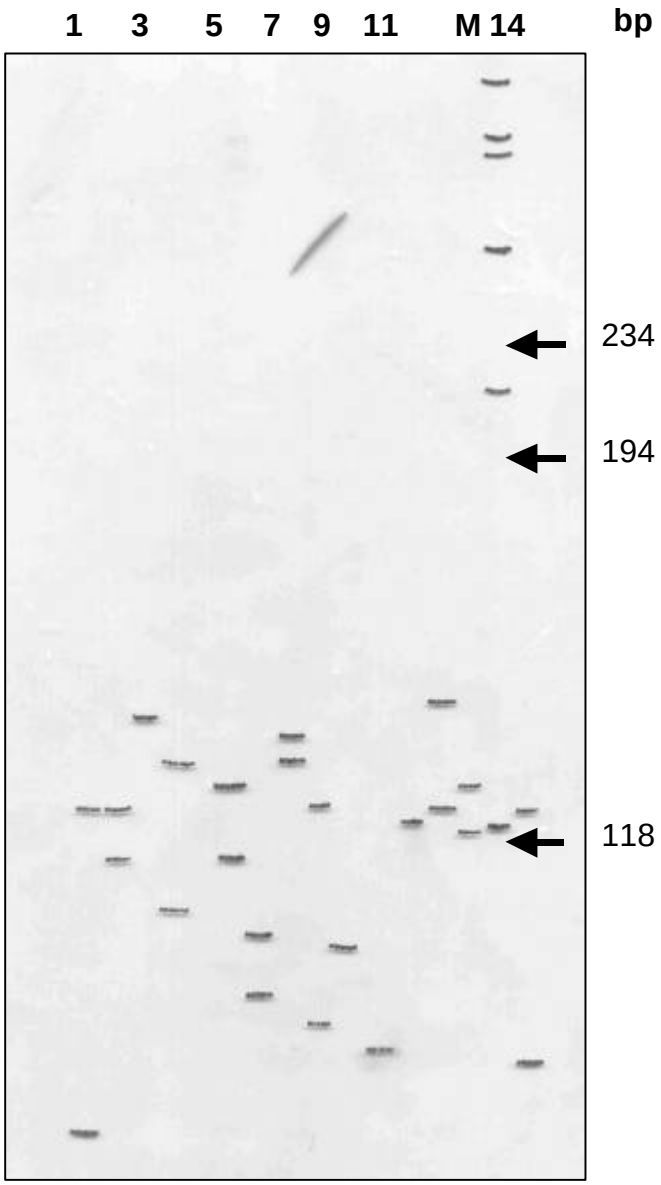
having sufficient power also failed to replicate the results of Wei *et al*. (MC Ginnis *et al*, 2001).

As stated above, our study using 53 schizophrenics and 50 controls indicated no association between the polymorphism and schizophrenia and these results are consistent with those of the Japanese group where the large family and population- based study did not lack statistical power. As we employed a case control design, the problem of generating false positive or false negative results cannot be ruled out. Further study using a larger sample size and investigation of more loci and SNPs in that region will be necessary to confirm the susceptibility at the NOTCH 4 loci for schizophrenia.

Analysis of the CAGRI locus

CAGR1 gene was found to be highly polymorphic in the population screened in the present study. Twenty alleles were present, none of the allele or allelic variant showed any difference in the frequency between patients and controls in the population as indicated in Table 17 and Fig 9.

Figure 9: Autoradiogram of amplified PCR products for CAGR1 gene



Lanes 1-14: PCR amplified products in the population studied;
Lane M: End labelled ϕX-174/Hae III digest MWM (bp)
resolved on 6% denaturing PAGE gel.

Table 17: Allele frequencies of $(CTG)_n$ repeats for CAGR1 gene in patients and controls.

Alleles	Frequency in patients	Frequency in controls
1	0.023	0.0
2	0.011	0.032
3	0.011	0.0
4	0.071	0.043
5	0.071	0.076
6	0.035	0.010
7	0.035	0.010
8	0.083	0.032
9	0.059	0.043
10	0.035	0.065
11	0.035	0.010
12	0.047	0.043
13	0.059	0.021
14	0.166	0.054
15	0.059	0.130
16	0.047	0.054
17	0.059	0.043
18	0.011	0.010
19	0.035	0.043
20	0.059	0.032

In the study carried out by Margolis *et al* (1996), the normal allele distribution ranges from 6 to 31 triplets. African and non-African populations have different allelic distributions. One allele with a repeat length of 46, that is 15 triplets longer than the other alleles, was noted. The authors speculated that a relationship could exist between repeat length and the phenotype of the individual (a typical movement disorder accompanied by bipolar affective disorder type II). However, the correlation between the two was difficult to ascertain.

As is apparent from Table 17 and Fig 9, none of the patients showed large repeats as seen in type II disorders. No association between Schizophrenia and the CAGR1 gene or any of its allelic forms was found in the population studied. Polymorphism studies of the CAGR1 gene revealed 20 alleles. In order to have any statistical validity for the association between schizophrenia and any gene such as CAGR1that has 20 alleles in the present study, the size of the population under study must be very high. It is, therefore, possible that with a population of 53 patients and 50 controls it would be premature to comment on the involvement of CAGR1 gene in susceptibility to schizophrenia.

Candidate molecules likely to be involved in schizophrenia from the neurodevelopment aspect

Distinct genes involved in the initial rostro-caudal and dorsal-ventral positioning encode transcription factors that share a DNA binding motif (homeobox) that allows them to act as master switches to direct the morphogenetic development of each segment of the embryo (McGinnis *et al*, 1984; Scott and Weiner,

1984; Gehring, 1987; Ingraham,1988). Protein products of homeobox genes bind to certain DNA sequences, enabling the activation or inactivation of other genes in spatially restricted domains of the embryo (Wilkinson *et al*, 1989; Lufkin *et al*, 1991). Alterations in any one of these genes might lead to greater changes in gross brain morphology than the subtle ones found in the schizophrenic brain. Mutation in a homeobox gene has been found to be associated with marked schizoencephaly, a gross deficit in telencephalic fusion (Brunelli *et al*, 1996). SCIP, a homeo domain protein of the POII family genes, is expressed specifically in layer V neurons of adult cortex and only begins expression in the population of neural cells after they have started differentiation (Frantz *et al*, 1994). Other POU III family members have been shown to be activated at various times before and after birth in discrete regions of the brain (Le Moine and Young, 1992; Mathis *et al*, 1992). Therefore, transcription family molecules that fit in this developmental temporal pattern are more likely to be candidate molecules in schizophrenia. However, so far there are no reports on any of the above genes being associated with the pathophysiology of schizophrenia.

Growth factors have been shown to regulate cell proliferation and differentiation by stimulating receptors on the cell surface of dividing cells. These include a class of molecules also termed as cytokines, which stimulate proliferation and differentiation of brain cell precursors (Gospodarowicz, 1983). In addition, there are members of the fibroblast growth factor (FGF) family, nerve growth factor, brain-derived neurotrophic factor, and the epidermal growth factor (EGF) family. Growth factors can exist in both membrane \ substrate bound and secreted forms and

accordingly exert contact dependent and diffusible effects (Carpenter and Cohen, 1990). Neurons compete for limited amount of target-derived growth factors, which are taken up by the axon terminals and transported back to the nerve cell body to promote neuron survival (Levi-Montalcini, 1987). Neuronal growth factors consist of many different families of growth factor peptides (Casper, 1996). The neurotrophic growth factor (NGF) family is the best example of peptides that induce process outgrowth, increase soma size and support the survival of central nervous system neurons, primarily cholinergic (Glass and Yancopoulos, 1993). Brain derived neurotrophic factor (BDNF) and neurotrophin-4\5 (NT 4\5) are to name a few examples of trophic factor family that have been shown to support the survival and process out growth of dopaminergic, serotonergic, glutamatergic and GABAergic neurons.

At molecular level, cell migration is thought to result from a combination of adhesive or repulsive action of many cell surface proteins and glycoproteins present on the neurons and along the migratory route. Three main families of adhesion molecules can be located on the cell surface. They are the immunoglobulin super family, the cadherins, and integrins (Jessel, 1991). Cell adhesion proteins exist in more than one form, which could be related to specific developmental or functional abilities. For example, neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily, has a heavily polysilated (PSA) form that has been involved in promoting the migration of immature neurons out of the adult neutrogena subependymal layer (Ono *et al*, 1994). A previous study suggests that lower number of neurons expressing the polysialic form of the NCAM in the schizophrenic brain might

lead to migration abnormalities, resulting in inwardly displaced neurons. Neuronal atrophy has been reported in schizophrenia (Rajkowska *et al*, 1998; Selemon *et al*, 1998) where neurons are observed to be misplaced, missized and disorganised (Benes *et al*,1998; Benes *et al*, 1991; Bebes and Bird, 1986). Significant neuronal reduction in the thalamus and nucleus accumbens (Pakkenberg, 1990; Young *et al*, 2000) and reduction in the mean volume of the nerve cell in the mesolimbic part are also reported (Bogerts *et al*, 1983; Falkai and Bogerts, 1986; Akbarian *et al*, 1993; Benes *et al*, 1998) which could be a result of migrational abnormalities.

Neuroscientists have hypothesized that either fewer cortico-cortical connections or dysfunctional connections may underlie the deficits in higher-level cognitive tasks that are manifested by most schizophrenic patients (Weinberger and Lipska, 1995). Many different proteins that are involved in the complex process of axon elongation these range from cell adhesion molecules and neurotransmitter receptors to microtubules and microfilaments (Kater and Guthrie, 1990; Reichardt *et al*, 1990). Limbic associated membrane protein (LAMP) is an example of a cell adhesion molecule critical to axon elongation. LAMP is highly expressed by the cortical and sub cortical neurons comprising the limbic system and has been shown to promote selective neurite outgrowth from limbic cortical neurons (Levitt, 1984; Pimenta *et al*, 1995).

Another molecule integral to normal axon outgrowth and targeting of a more general nature is growth-associated protein 43 (GAP-43). It is essential for growth cone migration, and is localized

subcellularly to the inner leaflet of the presynaptic membrane (Skene, 1989; Shea *et al*, 1991). GAP- 43 mRNA, and it's protein have been found to be abundantly expressed in neurons that reside in areas of adult human brain. The areas containing these neurons are thought to be highly plastic, such as limbic and prefrontal cortices (Neve *et al*, 1987; 1988; Benowitz *et al*, 1989) indicating the role of GAP-43 in modulating neuronal connections later in life. Interestingly reduced levels of GAP-43 mRNA have been found in the adult schizophrenic prefrontal cortex (Geethabali *et al*, 1996).

Thus a number of genes involved in various stages in neurodevelopment and neurotransmission, or other elements that can indirectly affect these functions can be considered as candidate genes for study from the neurodevelopment aspect. Schizophrenia exhibits a wide range of symptoms, and heterogeneity. Due to its speculative aetiology it seems likely that in different ethnic groups different genes play a major role in bringing about the susceptibility to schizophrenia. For example, in a population where the caloric intake is high, the diet is not rich in Vit E and C (elements that are high in antioxidants) and the environment is stressful, generation of excess of free radicals is observed. Under the circumstances oxidative stress enzymes could be playing a major role along with the genes / proteins involved in the neurotransmission cascade. Detecting this would be possible by subgrouping the patients on the basis of caloric intake, stress and response to neuroleptics before data analysis. Likewise, a subgroup of patients, "early onset" is more likely to have anomaly in the genes critical from the development aspect and could pose a liability to schizophrenia. Analysis of the data after sub grouping

the patients was not carried out in the present study due to the small sample size. It would, however, be of interest to note if association exists between any of the genes involved in neurodevelopment in the early onset patients.

Though no correlation was found in amplified trinucleotide repeats in the genes analysed in the present study, it would be worthwhile investigating if SNPs in NOTCH4, CAGR1 BDNF, GAP and other candidate proteins are involved in bringing about susceptibility to schizophrenia.

Chapter 4

APOPTOTIC REGULATION IN SCHIZOPHRENIA[2]

[2] The content of this chapter is a part of a full length paper to be communicated to Psychiatry Research

INTRODUCTION

Aetiology of schizophrenia has remained elusive. The neurodevelopmental hypothesis states that schizophrenia is acquired through early life neurobiological insults that produce permanent brain deficits which manifest as psychosis in early adulthood (Weinberger, 1987). However, a neurodegenerative hypothesis has also been proposed, based on clinical grounds, the protracted period of symptomatic dormancy and the progressive deterioration that follows the first episode of psychosis (Lieberman, 1999; McGlashan, 1988). During brain development, neurons initially send out exuberance of connections, which are eventually pruned (Cowan *et al*, 1984). Pruning of connections can result from the actual death of neurons and reflects the failure of competitive survival for connections. The ability to maintain connections with other neurons or postsynaptic sites is necessary for long-term neuron survival (Purves and Lichtmen, 1985). A reduction in trophic support to a neuron is thought to result not only in synaptic pruning, but also in the initiation of the so-called programmed cell death of neurons (apoptosis), a cellular process involving a sequence of cellular and genetic steps ultimately leading to DNA fragmentation (Steller, 1995). Role of apoptosis in the pathophysiology of schizophrenia, as related to neurodevelopmental insults has also been hypothesized (Catts and Catts, 2000; Margolis *et al*, 1994; Woods, 1998) A disregulation of apoptosis could underline both, that is limited neurodegeneration in concert with a neurodevelopmental disorder.

Apoptosis or programmed cell death

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. The survival of multicellular organisms depends on the function of a diverse set of differentiated cell types. Once development is complete, the viability of the organism depends on the maintenance and renewal of these diverse lineages. Within vertebrates, different cell types vary widely in the mechanism by which they maintain viability over the life of the organism. There is a type of cell death occurring in the nervous system that has been referred to as programmed, developmental or naturally occurring cell death. This type of cell death, that involves the loss of a significant numbers of neurons in a given population, often, occurs at a relatively late stage in maturation. Studies carried out by Viktor Hamburger and Rita Levi- Montalcini in the late 1940s and 1950s of normal and experimentally produced cell death in the chick embryo lead to the recognition of massive normal neuronal death during development (Hamburg and Levi-Montalcini 1949).

Morphological modes of cell death

Kerr and Wyllie have distinguished two general types of morphological changes accompanying cell degeneration (Wyllie *et al*, 1980; Wyllie, 1981; Kerr *et al*, 1987) One type referred to as necrosis is characterized by cellular oedema and culminates in the rupture of plasma and internal membranes and leakage of cellular contents. Necrosis generally occurs in pathology or following injury. The characteristics of necrotic cell death are consistent with a failure in osmotic regulation that may be triggered by a loss of cellular energy supplies. Second type of degeneration is apoptosis, named after the Greek term, referring to the naturally

occurring seasonal loss or falling of flowers, and involves a progressive contraction of cell volume and wide spread chromatin condensation but with the initial preservation of the integrity of cytoplasmic organelles. The affected cells separate into membrane-bound fragments that are rapidly phagocytosed by adjacent cells. Pyknosis is one prominent feature of apoptosis. Apoptosis is characteristic of normal tissue turn over, embryonic cell death, and metamorphosis.

In some systems, excitotoxicity and free radicals function as upstream initiators of programmed cell death. Though ischaemic injury-induced cell death has traditionally been characterized as necrosis, morphological and biochemical evidence of apoptosis have been well documented in experimental animal models of ischaemic brain injury. There is evidence of caspase activity in ischaemic brain (Namura *et al*, 1998). Caspase-11 is specifically induced by ischaemic injury. (Kang *et al*, 2000)

Biological roles of neuronal death

The death of neurons during development may serve a variety of adaptive functions, depending upon the age when death occurs, the species, neuronal type, and regions of the CNS. In the early stages of development, it may serve the purpose of eliminating defective cells, shape the gross morphology of regions of the nervous system and create morphologically permissive environment for axonal growth (Kallen, 1965; Cuadros and Rios, 1988; Hankin *et al*, 1988). Later, neurons may die after having served important but transient functions, like providing cues for early axonal projections (McConnel *et al*, 1989; Bate *et al*, 1981), serving as temporary targets for early afferents (Luskin and Shatz,

1985; Marin-Padilla, 1978) or mediating early functional (behavioral) requirements of developing animal. The massive loss of cells that occurs during more advanced stages of neuronal differentiation in vertebrates serves two major roles namely quantitative adjustments or numerical matching of interconnecting populations of neurons and the elimination of projections that is in some sense incorrect or aberrant. Error correction is an attractive hypothesis since it provides a plausible adaptive rationale for the death of developing neurons (Lamb *et al*,1988, Clarke *et al*,1981). The mechanisms that mediate the selective loss of neurons with erroneous projections remain to be determined. Neuronal death appears to have evolved to mediate a wide variety of adaptive functions during the development of the nervous system. Once excess numbers of neurons are produced, they become available for many different purposes. Perturbations of cell death in these different situations may result in the aberrant development of the structure and function of the nervous system.

Key molecules involved in neuronal apoptosis.

Mammalian apoptosis is regulated by the Bcl-2 family of proteins, the adaptor protein Apaf-1 (apoptotic protease - activating factor1) and cysteine protease caspase family. Neurons share the same basic apoptosis programme with all other cell types. However, different types of neurons and neurons at different developmental stages express different combinations of Bcl-2 and caspase family members, which is a way of providing the specificity of regulation.

PI(3) K-Akt pathway and Map kinase pathway are the two pathways for survival and both converge on the same set of proteins Bad and CREB to inhibit the apoptosis programme.

Induction of apoptosis due to trophic factor withdrawal

Neurotrophins and membrane depolarization activate signal transduction pathways that suppress apoptosis. However, what triggers the activation of apoptosis in the absence of survival signals remains to be understood. The removal of NGF results in a decrease in MAR kinase and PI93 K activities along with increased production of reactive oxygen species, decreased glucose uptake and decreased RNA and protein synthesis.

Although protein and RNA synthesis are significantly reduced in the early stages of sympathetic neuronal cell death, cell death cannot occur in the presence of inhibitors of protein and RNA synthesis, indicating that synthesis of certain pro apoptotic molecules is necessary.

The point of no return in the apoptotic pathway is at, or down stream of caspase activation, and suggesting that under certain pathological conditions the inhibition of caspase is sufficient to block neuronal death.

Implications of apoptosis in schizophrenia.

The massive cell death that occurs in many populations of the vertebrate neurons is a fundamental aspect of development. It follows that any perturbation, genetic or environmental in origin, that alters the survival of neurons to a degree that is outside the normal range of biologically acceptable variation will be maladaptive. In schizophrenic individuals, neuroimaging studies

demonstrate increased ventricular size, (Lawrie & Abukmail, 1998) smaller temporal lobes, (Dauphinais *et al*, 1990) and cortical reduction (Zipursky *et al*, 1994, 1998) with no evidence of glial scarring.

Neuronal atrophy with unchanged cell numbers, missized and maldistribution of neurons could also be indicative of apoptosis (Akbarian *et al*, 1993). This could have occurred due to the loss of certain neurons induced to die during developmentally appropriate stage resulting in maldistribution. In addition, it is likely that the neurodevelopmental deficit is related to reduced proliferation and migration contributing to the above-mentioned changes. Bcl-2, an antiapoptotic protein exerts a powerful neuroprotective effect on neuronal cultures. Cells over expressing Bcl-2 demonstrate considerable resistance to a variety of apoptotic insults (Zhong *et al*, 1993). Reduction in cortical Bcl-2 levels has been reported in schizophrenic individuals. Lower levels of Bcl-2 protein may signal neuronal vulnerability to apoptotic stimuli and to neuronal atrophy. (Jarskog L. F. *et al*, 2000).

METHODS AND MATERIALS

Experimental Strategy

Biochemical insults like hypoxia that lead to birth complications, toxic insults resulting from free radical generation, viral infection and ischaemia that are implicated as stimuli in abnormal neurodevelopment in schizophrenia are found to activate apoptosis pathways (Charriaut Marlangue *et al*, 1996; Thompson, 1995). These insults may result from biochemical or genetic accidents that might trigger apoptosis leading to neurodegeneration or abnormal neurodevelopment. In addition,

conventional treatment (antipsychotics like haloperidol) for schizophrenia may also trigger apoptosis. Hence, we wanted to investigate whether apoptosis occurs in patients with schizophrenia. Lymphocytes have several neurotransmitter receptors like neuronal cells and their molecular machinery is identical to that in the brain, though the physiological responses may vary. Therefore, experiments were performed with peripheral blood mononuclear cells (PBMC) to assess for apoptosis in schizophrenia.

Subjects

Fifteen established schizophrenia patients were recruited from Poona Hospital (Pune). Controls, matched for sex and age, were recruited from staff and students working at National Chemical Laboratory and National Center for Cell Sciences (Pune). All patients satisfied the DSM 1V criteria for schizophrenia. At the time of study, all patients were under atypical psychotic drug treatment. Informed consent was obtained from all the individuals participating in the study.

Assessment of apoptosis

Five ml of anticoagulated venous blood was collected and mononuclear cells were separated by layering on Ficoll - Hypaque. Briefly, 5.0 ml of blood was layered gently on 3.0 ml of Ficol - Hypaque and centrifuged at 3000 rpm, for 10 min at RT. The buffy coat containing the mononuclear cells was collected. Cell counts were taken using a haemocytometer and 10^6 cells/ ml were used for further analysis. Apoptosis was monitored by measurement of DNA content of the nuclei labelled with propidium iodide (FACS) and by TUNEL assay.

Becton Dickinson procedure for DNA Analysis

PBMCs (10^6 cells excluding tryphan blue) were washed in PBS and centrifuged at 2500 RPM for 10 min. Cells were fixed in chilled ethanol (70%), centrifuged at 2500 RPM for 10 min and the pellet was resuspended and incubated with RNAase A (5 mg/ml) for 10 min at RT. Propidium iodide (50 mg/ml) was added and cells were incubated at room temperature in dark for 30 min. Analysis was performed using 488 nm argon laser with FACS vantage (Becton Dickinson USA) The stained cells were analyzed for hypodiploidy as a marker for apoptosis. PBMCs were processed immediately after separation and adequate precautions were taken to ensure that there was no loss of cells.

In situ staining for DNA breaks

TUNEL assay which detects apoptotic cells using terminal deoxynucleotidyl transferase (TdT) to directly label the ends of broken DNA strands was performed for in situ detection of apoptotic cells. TUNEL stands for TdT- mediated dUTP – biotin nick end labeling and relies on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the exposed 3' – OH ends of DNA followed by synthesis of a labeled polydeoxynucleotide molecule. Biotinylated deoxyuridine is incorporated in to the sites of DNA breaks. The signal is amplified by avidin–peroxidase, enabling identification of PCD by light microscopy. Apotag R (Intergen kit) was used for in-situ detection for DNA breaks.

Cytospin preparation for in-situ staining:

PBMCs were washed in PBS and resuspended in 1% paraformaldehyde incubated at RT for 10 min. A monolayer of106 cells were fixed on each slide using Cytospin at 800 rpm for 10min.

At this stage, the slides can be stored at – 40 o C or processed immediately.

Peroxidase staining of cells:

PBMCs were stained and processed according to the manufacturer's instructions. In brief, after quenching the cells in 3% H_2O_2 slides were washed in PBS twice for 5 min each and equilibrated for 10sec in the equilibration buffer at RT, followed by incubation in working strength TdT at 37^o C for 1hour. Slides were then incubated in anti-dioxigenenin peroxidase conjugate for 30 min at RT in a humidified chamber. After washing the slides in PBS they were incubated in working strength peroxidase substrate for 3-6 min for colour development. Finally, the slides were counter stained in 0.5% (W:V) Methyl Green for 10min at RT, mounted and scanned for apoptotic cells.

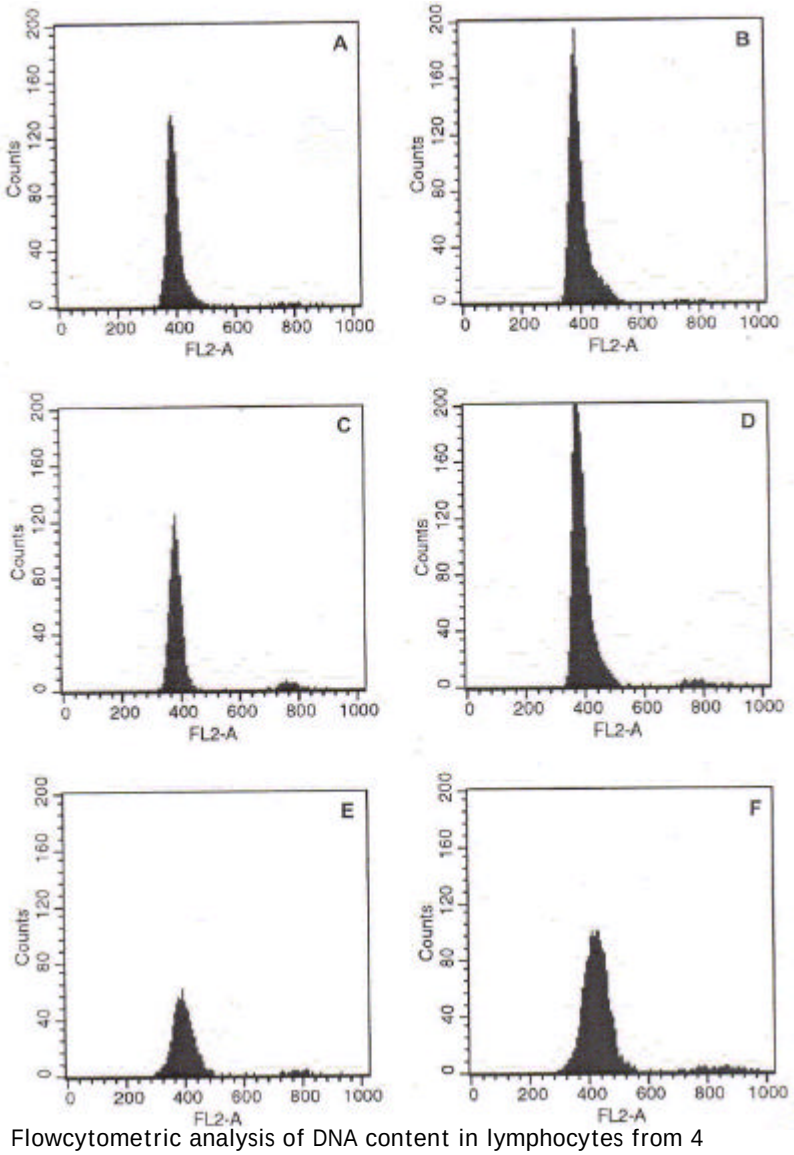
RESULTS AND DISCUSSION

Quantification of apoptotic cells by FACS

Flow cytometer can quantify the proportion of cells in various phases with fluorescent DNA dyes that quantify the fluorescent intensity of the dye. In addition to the emitted light, the flow cytometer also analyses scattered light from the cells passing through the laser beam. Forward scatter and light scatter indicate cell size and granularity or density, respectively. Since apoptotic cells typically decrease in size, the cytometer is able to detect apoptotic cells as smaller, less fluorescent entities. Thus in a DNA cytogram, apoptotic cells will give a fluorescence peak that is completely separated from the normal G1 peak at approximately 50% of the G1 peak fluorescence. Fig 10 represents

Flowcytometric analysis of DNA content in lymphocytes from 4 schizophrenic patients (A, B, C, D) and 2 normal healthy subjects (E,F). A single peak for G1 around 400 was obtained after analysis for 10,000 single events. Excitation was carried out at 488 nm line of an argon laser and detected at 620–700 nm. None of the samples analysed demonstrated hypodiploidy or sub- Go/Gi peaks indicating that the samples analysed did not show apoptosis.

Figure 1: Flowcytometric analysis of DNA content of nuclei labelled with propidium iodide.



Flowcytometric analysis of DNA content in lymphocytes from 4 schizophrenic patients (A,B,C,D) and 2 normal healthy subjects (E,F). Cell cycle distribution and assessment of apoptosis was done by plotting the intensity of propidium iodide fluorescence which reflects the DNA content on X axis and the relative cell number on the Y axis.

In-situ staining for DNA breaks

Slides were screened for the incorporation of labelled UTP in the nuclear material. Absence of incorporation indicated by negative signal for peroxidase staining confirmed the lack of apoptosis using in-situ TUNEL assay.

Reports demonstrating higher neuronal density, reduced neuronal size in the absence of glial scarring in the cortex of affected individuals, neuronal maldistribution and enlarged ventricles are indirect evidences for apoptosis. Lower levels of Bcl-2 found in schizophrenic patients could be the cause of neuronal atrophy and reduced axodendritic branching. This initial deficit accompanied by oxidative stress could result in triggering the apoptotic machinery during neuronal pruning. Increase in the levels of Bcl-2 in schizophrenic patients, treated with antipsychotic drugs has been reported (Jarskog *et al*, 2000). The reason for not detecting apoptosis in our patients could be the fact that most of our patients were medicated with atypical drugs. Medicated patients show lower oxidative stress indicated by lower lipid peroxidation as compared to unmedicated patients. (Arvindakshan *et al* In Press). Atypical neuroleptics reduce oxidative stress based on lower lipid peroxidation and increased membrane phospholipids (Arvindakshan *et al*, In Press). Atypical neuroleptics have also been shown to induce growth factors like BDNF and NGF in rat brain and in cell cultures. These neurotropic factors are known to protect neuronal cells against apoptosis by inducing Bcl-2 expression. Both BDNF and Bcl-2 expression are reduced in schizophrenics, however treatment with atypical antipsychotic drugs may lead to increased expression of BDNF and Bcl-2, which together may protect against apoptosis. In conclusion, our data

indicates no apoptosis occurring in schizophrenic patients undergoing antipsychotic treatment and this emphasizes the need to study apoptosis in first break drug naïve patients.

GENERAL DISCUSSION

INTRODUCTION

Schizophrenia is a complex disorder where genetic as well as environmental factors have been considered to play a critical role. Genetic studies carried out so far indicate that more then one gene is likely to be involved and it may be a multi gene\ polygenic disorder, where one or few genes may play a major role and other genes may have minor roles. Considering the fact that incidence of schizophrenia is 1%, a model involving several genes is easier to reconcile with the fact that schizophrenia persists at a higher rate in the population despite being associated with reduced reproductive fitness. However, it shows increased incidence to family history and anticipation. Dynamic mutations or the change in size of the repeat unit with successive generations have been found to be involved in several neurodegenerative disorders. Schizophrenia, however, is not considered as a classical neurodegenerative disorder and the progressive deterioration observed may be the result of defective neurodevelopment.

MY HYPOTHESIS

Considering the approach of trinucleotide repeats and the involvement of multiple genes, I wanted to test the hypothesis that the presence of amplified trinucleotide repeats within (coding region) some (Candidate) genes could predispose an individual to develop the illness.

UNIQUENESS OF THE POPULATION

Though the case control studies in my work involved a small sample size, the population studied was unique such that, the patients and controls were of the same ethnic origin. In India, we do have different religions, casts and there are areas or pockets where consanguinity is observed, most of the Christians and Muslims are converted hence the roots of most of the apparently diverse religions are common. In spite of the change in diet that occurred over the years, it does not differ much in the population that I have studied.

NUCLEOTIDE POLYMORPHISM IN SIX GENES EXPRESSED IN THE BRAIN

Diffused and heterogeneous pathology and physiology, found in several brain areas, particularly, the limbic, diencephalon and prefrontal cortex even at the onset of illness indicates a neurodevelopmental defect. A lesion present early in life may influence the normal brain maturation events that may precipitate psychopathology later. This lesion could be caused due to defective gene(s) or altered expression of a normal gene(s) due to stressful environmental factors. To test this hypothesis, genes important from the neurodevelopment aspect were included as candidate genes. Earlier results indicate that long repeats (50 repeat units) like those involved in other neurodegenerative disorders are not found in schizophrenia but the involvement of moderate repeats cannot be ruled out. Nucleotide polymorphism studies were carried out in the six genes expressed in the brain. One gene showed a monomorphic pattern and hence was not informative in the present study. Of the five genes that were polymorphic, heterozygous condition for gene U80738 was found

to be statistically higher in control individuals than the patient population. U-80738 is homologous to the nuclear matrix transcription factor and higher frequency for the heterozygous allele gives the heterozygote advantage over the homologous condition predominantly found in the patients. Though the controls used in this case control study were of the same ethnic origin, replication of the study using a higher population size or family based trios needs to be carried out to confirm the same.

Most of the genes studied so far have failed to show consistent association with the change in cohort, as was reported in case of HsKCa 3 and NOTCH 4 genes. In population based case control studies it is important to check that the sample studied has sufficient power, control individuals are matched for age, sex, and ethnic origin and association if present is not due to population stratification.

As environmental factors play a major role it is likely that different genes are involved in different populations or groups. The heterogeneity observed could be due to the changing environmental parameters; hence it would be important and useful to analyze the data by subdividing the patient population on the basis of various environmental effects. For instance stress as a parameter that affects cellular oxidative stress, generated on account of the free radicals produced due to psychopathological and often high caloric intake in patients. The antioxidant defence like Glutathione peroxidase, Superoxide dismutase & Catalase & protective action of dietary antioxidants like vit C & vit E helps to maintain a balance between the two. If this balance is not maintained excess of free radical generation could lead to

degradation and hence depletion of essential fatty acids in the membrane, enzyme inactivation and DNA damage. Increased oxidative stress indicated by higher lipid peroxidation has been reported in schizophrenia. When the genetic make up of the individual shows vulnerability (enzyme disfunction at the genetic or expression level), and environmental factors are conducive (high stress) an individual is more likely to succumb to the disorder. Thus in a population where caloric intake and stress is high (leading to more free radical generation) the enzymes and proteins involved in scavenging free radicals, those involved in the synthesis of essential fatty acids or having a direct effect on DNA could play an important role and exert a major gene effect and show association. When studying genes involved in neurodevelopment, segregation of the patients on the basis of early onset or late onset would be necessary when carrying out multiple testing with respect to various parameters.

To assess the role of involvement of trinucleotide repeats in schizophrenia, multiple genes that could be involved in playing a role as candidate genes have to be studied and evaluated on a large sample population or family based trios taking in to account the biochemical parameters that could be affected due to environmental factors while carrying out the analysis. Subdividing the patient population on the basis of sex, neuroleptic responders and nonresponders early or late onset depending upon the genes under study might prove to be useful.

LACK OF APOPTOSIS IN THE POPULATION STUDIED

Neuroimaging and postmortem studies are indicative of neuronal atrophy and neuronal misalignment. Biochemical insults like birth

complications leading to hypoxia, toxic insults resulting from free radical generation, viral infections and ischaemia that are implicated as neurodevelopmental insults might also be involved in triggering apoptotic pathways leading to neurodevelopment in concert with neurodegeneration. PBMCs from schizophrenic individuals analyzed did not demonstrate hypodyploidy or sub-G0\G1 peak. Lack of apoptosis was confirmed using in-situ TUNNEL assay. Most of our patients recruited for this study were treated with atypical psychotics. Reports indicate that medicated patients particularly patients treated with atypical neuroleptics show lower oxidative stress as compared to unmedicated individuals based on lower lipid peroxidation,. In addition, growth factors like BDNF and NGF are induced in the presence of atypical neuroleptics. These in turn may protect the neuronal cells against apoptosis. It would be of interest, therefore, to assess the apoptotic levels in a larger population wherein the patient population include unmedicated and those treated with both typical and atypical drugs.

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