1.1 Introduction:

The global market for drugs for the central nervous system (CNS) is greatly underpenetrated and would have to grow by over 500% just to be comparable to the global market for cardiovascular drugs (Pardridge W.M.-2002). **Brain Tumors and Gliomas** are one of the most deadliest disorders with more than 80 % mortality rate. A glioma is a type of tumor that starts in the brain or spine. It is called a glioma because it arises from glial cells. The prognosis for patients with high-grade gliomas is generally poor, and is especially so for older patients. The cure and management rate for brain tumor is expected to be much lower in the developing countries like India. A study conducted in the Tata Memorial Hospital provided a one-year demographic data and relevant tumor-related information on all 656 patients registered in the Neuro-oncology Clinic. Gliomas constituted 38.7% (254 cases) of CNS tumors, with high-grade gliomas comprising 151 (59.5%) and low-grade gliomas 79 (33.1%) cases (4).

Chemotherapy lacks efficacy in most histological types of primary human brain tumors and has, for most types, failed to improve outcome for patients. The unsatisfactory results with chemotherapeutic intervention in these cancers have been chiefly attributed to tumor-cell resistance (Bredel M. and Zentner J.-2002). Moreover, multidrug resistance gene 1 (MDR1) mediated resistance to chemotherapeutic agents is also becoming one of the major obstacles for the therapy of various cancer types (Rein D.T. et al-2011). Overall, the currently available chemotherapeutic and radiotherapeutic agents are inefficient for primary brain tumors which depend on a number of resistance mechanisms (Parkinson J.F. et al-2008 and Sarkaria J.N. et al-2008). Therefore, the treatment of chemoresistant brain cancers by **gene therapy** looks promising.

The **p53 gene** plays a vital role in the way a tumor responds to chemotherapy making the status of the p53 gene crucial for cancer therapy. The biological ability of a cell to enter the apoptotic pathway in response to DNA damage is determined by the p53 gene. However, in about 50% of human cancers, the p53 gene is defective due to mutation and hence, fifty percent of patients currently do not benefit from standard cancer chemotherapy. The p53 gene is a tumor suppressor gene, i.e., its activity stops the formation of tumors. The p53 gene has been mapped to chromosome 17. In the cell, p53 protein binds DNA, which in turn stimulates another gene to produce a protein called p21 that interacts with a cell division-

stimulating protein (cdk2). When p21 is complexed with cdk2 the cell cannot pass through to the next stage of cell division. Mutant p53 can no longer bind DNA in an effective way, and as a consequence the p21 protein is not made available to act as the 'stop signal' for cell division. Thus cells divide uncontrollably, and form tumors.

The gene therapy, conceptually, requires the introduction of a missing or defective gene into the cell nucleus. In other words, gene therapy works on the basic concept that the delivery of polynucleotides to the cells will alter the expression of given protein resulting in therapeutic benefit. Gene therapy involves delivering polynucleotides such as DNA, RNA, anti-sense ologonucleotides and small interfering RNA either locally or systemically. Gene delivery to remove or replace the mutated gene and insert new gene in defective cells can only be the way out to treat the diseases completely.

For gene transfer to be successful, the foreign gene must cross the outer membrane of the host cell and be transported to the nucleus. But as DNA is a long, slender, hydrophilic and high molecular mass poly-anionic molecule having micrometer dimensions, broad application of the naked DNA cannot travel successfully to the nucleus on its own and hence a carrier/vector is needed to mediate gene transfer for gene therapy. Therefore, there are two essential components in current gene therapy - an effective therapeutic gene that is required at a target site and an efficient and safe delivery system that carries the therapeutic genes to a specific target tissue or organ. Hence, the development of carriers capable of addressing the issues related to gene delivery could render gene therapy as a general treatment for many diseases. Since gene delivery is a multi-step process, an appropriate property of carriers would be needed to carry forward each step, rationally designed multifunctional vectors could overcome a series of extra and intra-cellular barriers at molecular level (Gao X. et al-2007).

Since the conception of the gene therapy, efficient and safe delivery of therapeutic genes to target cells remains the major concern. Viral vectors are usually efficient delivery agents owing to their evolution in transfecting host cells, but they are marred by safety concerns in the clinical setting. Non-viral carriers are relatively safer but are less efficient in terms of transgene delivery. Efficient gene delivery holds the key for therapy of genetic disorders and other conditions, such as cancer and AIDS, which are unresponsive to currently available conventional drug therapies. Researchers across the globe are trying to develop a

non-viral gene carrier system that could have comparable transfection efficiency to viral carriers without compromising their inherent safety. High molecular weight **polyethylenimine (PEI)** has been reported to be effective for gene delivery, since plasmid DNA can be delivered to the cytoplasm via endosomes due to the proton-sponge effect of PEI (Godbey, W. T. et al-1999). However, high molecular weight PEI has not been clinically employed due to its non-specific cytotoxicity displayed against different cell types. On the other hand, low molecular weight PEIs are much less cytotoxic, but they are not efficient transfection agents due to limitations of DNA delivery into the cytoplasm. In an effort to improve the transfection efficiency of polymeric carriers, water-soluble lipopolymers (WSLP) were synthetized by combining lipidic components with the PEI. The PEI was expected to condense the plasmid DNA and enhance endosomal release due to its tertiary amines, while the lipid coating on plasmid DNA/polymer complexes may increase the permeability of complexes through cell membranes. A versatile class of gene carriers could be designed in this way by controlling the nature of the cationic backbone, lipidic substituent and extent of substitution. Substitution of branched PEI's primary amine groups is particularly attractive since it is amenable to a variety of chemistries.

The **polyallylamine 15 kDa (PAA15)**, a less investigated polycationic polymer**,** was also chosen to compare the effect of lipidic substitutent on transfection efficiencies. It is known fact that cationic polymers possessing primary amine groups are inefficient in transferring nucleic acids into eukaryotic cells, until they possess fusogenic or lysosomotrophic effect. PAA carries a strong positive charge, which makes it suitable to bind and package negatively charged DNA. It is a pH-sensitive polymer, extensively used in the pharmaceutical industry. PAA also contains non-titratable primary amino groups and lacks titratable secondary and tertiary amino function, which contributes to the buffering capacity. Hence, osmotic endosomal swelling is not induced by these polymers, leading to feeble DNA-polymer complex escape (Chen D. J. et al-2005). However, due to strong polycationic character PAA causes cytotoxicity and that has severely restricted its use as a gene delivery system. In order to reduce its cytocoxicity, Pathak A. et al have modified PAA with imidazolyl functions of polyallylamine and found that its transfection efficiency was increased and cytotoxicity was decreased significantly (Pathak A. et al-2007). Also, previously, PAA was modified with hydrophilic methyl glycolates and found that its ability

to mediate gene transfer into cells increased by several orders of magnitude. At the same time, it was observed that such glycolylation of the amine groups of polyallylamine decreases cytotoxicity (Boussif O. et al-1999).

Hydrophobic modification of low molecular weight (LMW) polyethylenimine (PEI) is known to increase gene transfection efficiency of LMW PEI (Teo P.Y. et al-2013). **Cholic acid (ChA)** was selected as a lipid substituent for manipulating the hydrophobicity of polymer conjugates because of its biocompatibility and other unique structural features. The bile acids and their derivatives are known to interact and destabilize the membranes (cell and endosomal membrane) owing to their amphiphilic characters. Cholic acid has three hydroxyl groups axially placed on one side to make it a facial amphiphile as opposed to head-to-tail amphiphile (Walker S. et al- 1996) and it has a steroid backbone in its structure. It is reported that steroid receptors are present on nuclear membrane (Rebuffat et al-2001) and recently it was shown that bile acids are natural ligands to farnesoid x receptor (FXR) (Hua T. et al-2000). Moreover, the bile acids and their derivatives are known to interact and destabilize the membranes (cell and endosomal membrane) owing to their amphiphilic characters.

In addition to the development of chemo-resistant cells within the tumor, the failure of chemotherapy to eradicate brain tumor has also been due to inadequate quantity of drug reaching the target site. Another challenge faced by CNS disorders is the Blood Brain Barrier (BBB), which acts as a major barrier for most hydrophilic substances and for larger lipophilic molecules (Pardridge W.M.-2002) in reaching the brain and has been the hurdle to overcome in brain delivery of therapeutic agents. The BBB has been called "the problem behind the problem" of CNS drug development (Fortin D. et al-2005). In current practice, delivery of the therapeutic gene to the brain requires drilling a hole in the head followed by insertion of the gene incorporated in a viral vector. However, craniotomy based delivery does not enable the expression of the therapeutic gene widely throughout the brain or even to relatively localized area such as a brain tumor, which could have a volume greater than several milliliters. Therefore, brain targeting technology is needed to be employed to enable the nanocarrier to traverse the BBB (W.M. Pardridge-2001).

Certain endogenous large-molecule neuropeptides such as insulin, transferrin, or lectin access the brain from blood via receptor-mediated transport (RMT) across the BBB. This transport is mediated by specialized ligand-specific receptor systems, including the

insulin receptor (IR) or the transferrin receptor (TfR), which are highly expressed on the capillary endothelium of brain. Hence, transferrin is widely used as a ligand for facilitating brain targeted delivery. We selected **transferrin (Tf)** as a brain targeting ligand owing to its utility and previous reports of its incorporation in polymer/plasmid DNA complexes.

Nanoplexes are nanocarriers prepared by polyelectrolyte complexation of anionic DNA and cationic polymer/lipid. Nanoplexes are very effective gene delivery systems and have low immunogenicity in comparison with viral vectors. Nanoplexes play an important role in targeted cancer therapy and an optimal delivery system provides an efficient and highly specific recognition of the target cells. Moreover, rapid internalization of the therapeutic gene into those target cells increases the cytotoxicity to cancer cells.

1.2 Aims and Objectives

The aim of the present research work was to develop nanoparticulate therapeutic gene delivery systems for targeting brain tumor using p53 tumor suppressor gene as the therapeutic agent and transferrin (Tf) as the ligand for targeting purpose. It was also aimed to synthesize novel polymer conjugates as safe and efficient nonviral gene carriers.

Thus, the present research was proposed to be carried out in four parts –

(1) to modify cationic hydrophilic polymers (PEI2, PEI25 and PAA15) by cholic acid (ChA) as a hydrophobic substituent to synthesize novel polymer conjugates (PEI2-ChA, PEI25-ChA and PAA15-ChA) and to determine their transfection efficiency;

(2) to use the most efficient polymer conjugate for delivery of pp53 (pDNA containing p53 gene) in mammalian cell lines by forming transferrin containing nanoplexes;

(3) to study transferrin containing nanoplexes for their brain targeting efficiency and

(4) to carry out tumor regression studies to judge the therapeutic potential of the developed brain targeted gene delivery systems.

Objectives of the Proposed Work

- Transformation of a plasmid DNA (containing p53 gene) into bacterial cells for replication, isolation & purification of the plasmid,
- Synthesis and characterization of polymer conjugates of cholic acid (ChA) with three different polymers (PEI2, PEI25, and PAA15)

- Evaluation and establishment of the synthesized polymer conjugates as efficient transfecting agents in different cell lines using suitable reporter gene
- Demonstration of the ability of synthesized conjugates to form nanoplexes with pDNA, incorporating transferrin (Tf) in nanoplexes and demonstrating brain targeting ability of such nanoplexes in vivo
- Demonstration of the ability of growth arrest and/or apoptosis by nanoplexes containing p53 in different cell lines using flow cytometry
- Assessing the tumor regression ability of transferrin containing nanoplexes of successful polymer conjugates with pp53 in a xenograft mice model with one of the tested cell line.

1.3 Hypothesis

It was hypothesized that ligand targeted nanocarriers containing therapeutic gene will provide more efficient and direct delivery of the tumor suppressor gene in brain tumors. Hence, successful formulation of such brain targeted delivery systems may give rise to suitable option for brain cancer by avoiding drawbacks of conventional chemotherapy.

1.4 Plan of the Work

1) To synthesize and characterize polymer conjugates of cholic acid (ChA) with three different polymers (PEI2, PEI25, and PAA15).
2) To form the competent cells of E.coli (DH-5α) and transform and isolate the pp53 (plasmid DNA containing p53 gene).
3) To evaluate the cytotoxicity of synthesized polymer conjugates and to demonstrate the transfection efficiency of nanoplexes in several different mammalian cell lines (including cell line for neuroblastoma) to establish them as efficient non-viral gene carrier.
4) To evaluate the binding capacity of synthesized polymer conjugates with pDNA and ability to form nanoplexes
5) To evaluate the ability of nanoplexes to protect the pDNA against activity of DNase I enzyme and stability against anion challenge.
6) To incorporate transferrin (Tf), a brain targeted ligand, into nanoplexes.

7) To demonstrate the ability of polymer conjugates to enhance the production of recombinant bone morphogenetic protein (BMP-2) in mammalian cell line.
8) To demonstrate the ability of growth arrest and/or apoptosis by nanoplexes containing p53 in suitable cell lines using flow cytometry.
9) To carry out the direct protein expression studies of nanoplexes with plasmid p53.
10) To demonstrate the brain targeting ability of transferrin containing nanoplexes using suitable technique.
11) To assess tumor regression study of transferrin containing nanoplexes of successful polymer conjugates with pp53 in a xenograft mice model.