Hypertension is a silent killer with rare manifestation of symptoms. It is responsible for 45% and 51% of global deaths due to heart disease and stroke respectively. People in low-income countries are largely affected with this risk factor due to larger population and poor health awareness among the masses. Lack of awareness about hypertension is the main reason because of which it remains undiagnosed till it leads to some serious complication by causing target organ damage. It has to be monitored on regular basis for proper well-being of an individual. Renin angiotensin system (RAS) is the most critical pathway involved in blood pressure regulation and any alteration in expression pattern of RAS genes (\textit{AT1R, ACE}) leads to high blood pressure. Of all the components of this pathway, Angiotensin converting enzyme (\textit{ACE}) and Angiotensin II type 1 receptor (\textit{AT1R}) genes play pivotal role. Conversion of Angiotensin I to Angiotensin II is catalyzed by ACE and Ang II is effector biological ligand for AT1R. All the blood pressure regulating functions of Ang II are mediated by AT1R which highlights the significance of this receptor. Several population based association studies targeting single nucleotide polymorphisms (SNPs) of \textit{AT1R} have been done in different populations with respect to hypertension and other cardiovascular disorders (CVDs) but with varying results. This non-reproducibility of results between different studies could be due to differences in ethnicity, age and other selection criteria used by each of these studies. It further highlights role of certain other unexplored but functionally significant phenomenon. Epigenetic mechanisms which include DNA methylation, histone modification and miRNA involvement can be among those unexplored medium responsible for such contradictions. Therefore, we took the objective of analysing the effect of promoter DNA methylation in \textit{AT1R} gene.

Role of DNA methylation on expression of cancer and other developmental genes is vastly explored but such methylation based studies in blood pressure regulating genes has only recently been started. Studies on rat models have found differential expression of RAS genes due to altered promoter methylation. Maternal low protein diet (MLPD) caused promoter hypomethylation based overexpression of \textit{AT1b} resulting in hypertensive offsprings (Bogdarina \textit{et al.}, 2007). Effect of MLPD on expression of other RAS genes (\textit{ACE} and Angiotensinogen, \textit{AGT}) was observed where hypomethylation of CpG island in \textit{ACE} promoter region altered expression level (Goyal \textit{et al.}, 2010). Administration of nicotine to pregnant rats caused
epigenetic programming of AT1a where decrease in promoter methylation enhanced AT1a receptor (Xiao et al., 2014). Similarly, heightened AT1a expression in spontaneously hypertensive rats (SHR) was related to promoter hypomethylation which increased blood pressure in SHR as compared to Wistar-Kyoto (WKY) rats (Pei et al., 2015). Thus, rat studies highlighted the role of methylation on expression pattern of blood pressure regulating genes. In continuation to rat studies, epigenetic regulation of human genes has also gained interest and difference in methylation pattern at global as well as individual gene level has been analysed. Global level of 5mC in DNA isolated from blood sample was found to be lower in hypertensive individuals compared to normotensives which suggest that even peripheral blood sample exhibits differences due to hypertension (Smolarek et al., 2010). In addition to global methylation pattern epigenetic control of certain selected genes namely, HSD11B2, ADD1, GCK, CYP11B2 was studied where promoter methylation pattern affected individual gene expression.

Seeing the functional relevance of promoter methylation on gene expression and results from rat studies, methylation pattern in promoter of human AT1R was targeted in present study. At the time of initiation of this work no report on human AT1R gene was present in literature. Though recently studies on promoter methylation of AT1R along with other selected genes have been reported (Sykes et al., 2015; Foy et al., 2015). AT1R promoter methylation along with other cancer associated genes was analyzed and methylation index was calculated for all studied promoters to associate promoter methylation of these genes with increased risk of development of Oral squamous cell carcinoma (OSCC) in patients with oral premalignant lesions (OPL) (Foy et al., 2015). Similarly, methylation pattern in intrauterine RAS was studied where gestation or labour showed no effect of methylation on expression of intrauterine RAS genes (Sykes et al., 2015). But none of these studies worked on the site specific methylation of AT1R promoter. However, methylation pattern at few selected CpG sites (n=8) within AT1R promoter targeting essential hypertension was recently reported (Fan et al., 2015b). But it has its own constraints as they targeted CpG methylation in short fragment rather than complete CpG island (CGI) lying within AT1R promoter and performed no expression analysis. Thus, present work becomes the first report to target methylation pattern of CGI present within AT1R promoter and study its functional activity. CpG Plot software predicted CpG island in
AT1R promoter region spanning exon 1. Functional role of this CpG island in promoter of AT1R (CpG.P.AT1R) and its effect on expression was targeted but due to non-availability of AT1R expressing cell line, reporter gene assay was performed. Despite enormous efforts, when amplification of targeted region was not achieved then it was commercially procured and sub-cloned in promoter-less reporter vector. Transfection of cloned construct in cell lines showed promoter activity of CpG.P.AT1R through reporter assay results. Role of methylation on this region was also found as methylated construct resulted in decreased expression of reporter gene as compared to non-methylated/mock construct. Reporter assay results were further confirmed through sequencing of mock and methylated construct where methylated CG sites were observed in methyltransferase treated construct affecting reporter gene expression. In addition to this, treatment of construct with increased concentration of demethylating agent prior to methyltransferase treatment rescued the reporter gene expression, validating the effects of methylation on targeted region. Further, the effect of enzyme concentration and duration of methyltransferase treatment on promoter activity was studied where increase in both enzyme concentration and duration of treatment separately, decreased expression of reporter gene to greater extent. This result so far becomes the only report showing direct functional role of CpG island in AT1R promoter and its alteration by methylation. From in-vitro results this study was carried to in-vivo experiments where methylation pattern of CpG.P.AT1R was compared between hypertensive (HTN) and normotensive (NTN) individuals. As per recent reports methylation pattern is influenced by diet regime where dietary nutrients like folate, choline, vitamin B12, betaine affect DNA methylation through 1-carbon metabolism (Choi and Friso, 2010; Anderson et al., 2012). Main metabolites of this pathway which alter DNA methylation includes S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). S-adenosylmethionine is primary methyl donor and S-adenosylhomocysteine is methyltransferase inhibitor. Thus any dietary supplement which affects the levels of these two metabolites within a tissue can change DNA methylation pattern. Targeting the role of diet and other risk factors for hypertension including physical inactivity, obesity, alcohol consumption and others, samples were collected from Chandigarh specifically (including tricity region) as people here follow luxurious lifestyle with more of sedentary routine. Clinical details and other relevant information (diet, physical activity, alcohol consumption and smoking) were taken from all the volunteers at time of sample collection. Isolated
DNA from collected blood samples was bisulphite treated for methylation study. Entire CpG.P.AT1R was covered using two overlapping primer sets and methylation pattern was compared through sequencing results of purified PCR products. No difference in methylation pattern at any CpG site was found among both groups. However, hemi-methylation at one CpG site was found in one of the hypertensive sample. Hemi-methylated site harboured binding site of Sp1 transcription factor but CpG methylation does not affect binding affinity of Sp1 (Harrington et al., 1988; Holler et al., 1988). Though Fan et al. studied 5 CpG sites in fragment of AT1R promoter and reported hypomethylation at first CG site (CpG1) in EH cases compared to that in controls. But in our study no methylation difference was observed among HTNs and NTNs at any of those CpG sites which were screened by Fan et al., 2015.

As in human samples no alteration in methylation degree or pattern was observed between hypertensives and normotensives, we screened the individuals for known functionally relevant SNPs and novel variations. Two of the SNPs of AT1R (rs5186 and rs422858) with functional significance were screened along with ACE (I/D) polymorphism. As additive effect of ACE (I/D) and rs5186 on diastolic blood pressure and myocardial infarction has been found (Henskens et al., 2003; Kaur et al., 2012) so this polymorphism was also selected for screening along with AT1R polymorphisms. Genotyping was performed using PCR-RFLP mainly and RFLP results were further validated through direct sequencing of PCR products.

a) rs5186 (A1166C) is the most widely studied of all known AT1R polymorphisms ever since Bonnardeaux et al. 1994 reported higher frequency of C\textsuperscript{1166} allele in HTN group. Like other SNPs, A1166C also shows mix of positive and negative association outcomes (Sugimoto et al., 2004; Lapierrre et al., 2006). Though C\textsuperscript{1166} allele is prominent in families with positive history (Wang et al., 1997; Shahin et al., 2014), it also shows gender specific association with hypertension (Liu et al., 2002; Stankovic et al., 2003). Though this polymorphism is studied in various populations but very few studies (with both positive and negative association outcomes) have been reported from India, especially for hypertension (Chandra et al., 2014; Singh et al., 2014, Patnaik et al., 2014a). No significant difference of this SNP was found in present study similar to negative reports from South Indian population (Ramu et al., 2011; Singh et al., 2014). In our study of limited samples we observed the base line frequency of C\textsuperscript{1166} as (0.063, Table 24) which was very different from the frequency
reported in South Indian population (0.40, Singh et al., 2014) but much similar (0.06) to the report from Kaur et al. 2012. However another study from north Indian population (Chandra et al., 2014) has reported positive association of C1166 allele with hypertension. The reason for difference in association outcome might be due to the reason that samples collected in that study were from one of the premier medical institute of North India and our sample set represents Chandigarh region specifically. This is further supported by almost similar match of allelic frequency among controls as reported by Kaur et al. 2012 which involves sample set from our region. In addition to this, difference in sample size and age criteria can also be a reason for observed difference in both studies.

b) rs422858 (A-214C): Not even a single report on this SNP was found from Indian population as per our literature search. This promoter polymorphism lies in the sequence which includes USF (Upstream stimulatory factor) binding site. Studies on this SNP with other promoter polymorphisms as a haplotype in hypertension (Wei et al., 2003) and myocardial infarction (Poirier et al., 1998) showed positive association with hypertension in Caucasians and with raised blood pressure as a haplotype in transgenic mice (Jain et al., 2013). Although we did not find any significant statistical difference between frequency of allele A among NTN (0.837) and HTN (0.833) but gender based segregation revealed slightly higher frequency in HTN males (0.873) and lower in HTN females (0.788) in comparison to respective NTN group (Table 24). Notably, the frequency of allele A in normotensive individuals was much higher (0.837) than that reported in Caucasians (0.74, Jain et al., 2013) indicative of population differences.

c) rs4340 [ACE (I/D) polymorphism] has been analysed in various diseases (Raza et al., 2014; Barauh et al., 2016; Javadi et al., 2016) including hypertension where DD genotype showed positive association with hypertension (Choudhury et al., 2012; Patnaik et al., 2014b), but studies with negative association also exist (Negi et al., 2015). Our study showed almost same allelic distribution among HTN and NTN but gender based segregation showed significant difference (p=0.003, Table 25) between both alleles in females where allele I was found to be more common among HTN group and allele D among NTN. Same was reported in a study from north Indian sample set where allele I was found to be associated with EH (Srivastava et al., 2012b) and DD genotype to be more common among controls (Singh et al., 2016)
which is in contradiction with other reports (Choudhury et al., 2012; Patnaik et al., 2014b) on Indian population.

Genotypic and allelic frequencies of all three SNPs were also calculated on basis of family history and age of onset. But no significant difference was found between two groups in any of the studied SNPs on such categorization. In addition to these categories, lipid profile and other lifestyle factors were also compared (Table 28). Lipid profile including TG, TC, HDL-C, LDL-C and VLDL-C showed no significant difference. However, higher percentage of NTN individuals with occupational sedentary activity and non-veg diet was observed but they were still normal as they had habit of morning/evening walk or running and were not regular or passionate consumers of non-veg diet. Thus, rare non-veg diet might have worked as protein supplement similar to what is provided in DASH diet (Dietary Approaches to Stop Hypertension). Such practice of physical exercise and diet regime might have prevented development of high blood pressure in NTN group which was further evident from lower BMI readings observed in this group. Statistically significant difference was found between Body mass index (BMI), Systolic and Diastolic blood pressure readings where all three values were higher among HTN individuals (Table 28). In addition to this, mean onset age of hypertension showed significant difference among familial and sporadic group (40.1±5.8 vs 45.6±5.9, \( p=0.03 \)) with onset of hypertension much earlier in familial group.

Search for novel variations in the exons of \( AT1R \) did not yield much in terms of novelty. Exon 1 was found completely devoid of any variation which could also be attributed to the fact that this exon is part of CpG island which constitutes \( AT1R \) promoter. Sequence analysis of exon 2 revealed existence of already reported SNP (A/G-rs2276736) in our sample set with a frequency of (G=0.66). This SNP was reported in relation to non-alcoholic fatty liver disease (NAFLD) where allele A was found to be protective against NAFLD in Indian population. In exon 3 neither novel nor any reported SNP was found to exist in this sample set. Exon 1, 2 and 3 altogether cover 397 bp region of \( AT1R \) (47 Kbp) but it is interesting to report that in our study this region is almost devoid of variations indicating involvement of additional yet undiscovered factors.