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Original Research Article

Regulation of Human Cathepsin L expression by "AAAT" repeats

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Abstract

Over expression and secretion of human cathepsin L, a lysosomal cysteine protease, is one of the major determinants for the gain in metastatic ability of cancer cells. However, in humans, this protease is regulated at multiple levels. In this study, we have explored the significance of a repeat region present in its alternate promoter. Sequencing this region in seven different cancer cell lines revealed the presence of either eight or ten repeats. Cell lines with ten repeats displayed higher cathepsin L mRNA and protein expression. Further, recombinant reporter constructs containing either eight or ten repeats upstream to luciferase were transfected in cell lines to analyze their transcription regulation abilities. We observed that the construct with ten repeats resulted in higher transcription, albeit in a tissue specific manner. This is the first report demonstrating the involvement of a tandem repeat element in regulating cathepsin L expression.

Keywords: UTR, IRES, CTSL, hCATL

1. Introduction

Human cathepsin L (CTSL) is a ubiquitously expressed lysosomal cysteine endopeptidase, majorly involved in intracellular protein turnover[1]. However, upon over expression in various pathological conditions, it is secreted into the extracellular milieu[2]. It is implicated in the pathology of almost all types of cancers, degrading the matrix proteins, thereby facilitating tumor invasion and metastasis[3]. Advanced tumor stage and grade has also been correlated with increased CTSL activity[4] and is indicative of poor prognosis[5][6]. Using various CTSL inhibitors, it has been demonstrated that metastasis by malignant cells can be blocked *in vitro*[7]. Also blockage of its secretion has been shown to decrease tumor growth and vascularity in nude mice[8]. Thus, regulating CTSL expression in cancer cells would play a pivotal role in controlling their metastasis.

Our lab is mainly involved in studying the regulatory mechanisms controlling CTSL expression in cancer. Cloning of the human *CTSL* gene revealed that it contains eight exons and seven introns and the first exon is not translated and is therefore the 5'UTR[9]. Two TATA less promoters encode for two different mRNA species, named as hCATL A and B. Alternative splicing in the hCATL A mRNA, generates three more splice variants, AI, AII and AIII. The AIII variant was first identified by us and was shown to be the most abundant mRNA species in cancer cells[10]. We have demonstrated that these splice variants can differentially regulate CTSL levels as they possess variable translational abilities and stabilities. The longest variant, hCATL A, was also shown to contain a functional IRES and it translated with the highest efficiency in cancer cells[11]. Recently, we also demonstrated the role of

promoter methylation on stage specific expression of CTSL in chronic myeloid leukemia and observed an increased CTSL expression in chronic phase as compared to accelerated/blast crisis patients[12].

2. Materials and Methods

2.1 Cell Culture

The human cell lines namely Chang liver (normal liver), HepG2 (hepatocellular carcinoma), Hs294T (melanoma), H520 (lung adenocarcinoma), SiHa (cervical cancer), U87MG (glioblastoma, p53 wild type) and U373MG (glioblastoma, p53 mutated) were purchased from NCCS, Pune, India. All the cell lines except Chang liver were maintained as adherent monolayers in DMEM media (Sigma-Aldrich, MO, USA) with glutamine, glucose and sodium pyruvate supplemented with 10% fetal calf serum and 20 µg/ml ciprofloxacin, in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The Chang liver cells were similarly cultured in MEM media.

2.2 Isolation of Genomic DNA

Total genomic DNA was isolated from cell culture flasks (70-80% confluent). The cultured cells were washed with ice cold phosphate-buffered saline (PBS) three times and then subjected to isolation of DNA by using genomic DNA purification kit (Promega, Madison, WI, USA). The integrity of the purified DNA was assessed by running an aliquot on 1% agarose gel.

2.3 Amplification and cloning of AAAT repeats in the *CTSL* promoter

A nested PCR based strategy was adopted to amplify the AAAT repeat region in the first intron (Figure 1A). A Primary PCR with total genomic DNA (100 ng) from various cell lines was performed using DR-17 and DR-10 as sense and antisense primers respectively. The primary PCR products were then used as templates to perform a secondary PCR using primers PS1 (sense) and SSC164 (antisense), thereby amplifying a 900 bp product. Finally, a 350 bp fragment was amplified with the secondary PCR product and internal primers PS2 (sense) and RPS2 (antisense). These amplicons were further cloned into the pGEMT Easy vector and sequenced (Microsynth, Switzerland).

2.4 Cathepsin L Enzyme Assay

Cells were washed twice with ice cold PBS and lysed in Tris HCl buffer (50 mM Tris HCl, pH 6.8; 150 mM NaCl; 10% Glycerol; 1% Nonidet P-40) followed by two freeze thaw cycles. The cell lysate was centrifuged at 10,000 g for 15 minutes at 4^0C to remove the cell debris. The CTSL activity in the clear supernatant was assayed using a synthetic fluorogenic substrate, CBZ-Phe-Arg-N-Methylcoumarin, as described earlier[13]. Fluorescence was recorded at an interval of one minute with excitation wavelength at 370 nm and emission at 460 nm. One unit of enzyme activity was defined as a unit change in fluorescent emission at 460 nm, with excitation wavelength at 370 nm, when enzyme was incubated with 10µM of CBZ-Phe-Arg-N-methylcouramin, at 30ºC for 1 minute, under conditions described above.

2.5 RNA isolation and Real time PCR

Total cellular RNA was isolated from the cultured cells using Tri reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The integrity of the isolated RNA was checked on formaldehyde agarose gel. 5 µg of total RNA was reverse transcribed using RevertAid™ M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania) and random hexamers according to the manufacturer's protocol. An aliquot containing 200 ng of the total cDNA was subjected to PCR using primers specific for CTSL (DR-16 and SSC-30) and GAPDH (7GF and 8GR) on a Bio-Rad I-cycler (Bio-Rad, Hercules, CA, USA). PCR reactions were carried out in a final volume of 25µl containing 1.5 mM Magnesium chloride, 20µM of each of the primers, 0.2 mM dNTP mix, 1U Taq Polymerase (Invitrogen Corporation, Carlsbad, CA, USA), 1X PCR Buffer and 1X SYBER green (Invitrogen Corporation). PCR conditions comprised 35 cycles of denaturation at 94^0C for 30 sec, annealing at 60^0C for 45 sec, extension at 72^0C for 1 min and fluorescence recording at 80^0C for 30 sec. Similarly GAPDH served as internal control. Melting curve analysis confirmed no primer–dimer formation for CTSL and GAPDH under the above-mentioned conditions. The expected sizes of the PCR products were confirmed by agarose gel electrophoresis. Cycle threshold [Ct] values were calculated for each PCR and relative fold abundance was calculated using $2^{-\Delta Ct}$ method.

2.6 Western blotting

Cultured cells were washed twice with ice cold phosphate buffered saline and lysed in RIPA buffer (50 mM Tris / HCl pH 7.5, 1 mM EDTA pH 8.0, 1% NP-40, 150 mM NaCl, 10 mM MgCl2, 10mM NaF, 1.0 µg/mL protease inhibitor cocktail). Cell lysates containing equal amounts of total protein (~ 60µg) were resolved on 12% SDS PAGE and transferred on to a 0.45µm (pore size) nitrocellulose membrane (MDI, India). CTSL was detected by incubating the blots with a monoclonal anti-CTSL IgG (Sigma Aldrich) followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). The protein bands were visualized using premixed 5-bromo-4-chloroindol-2-

yl phosphate / Nitro Blue tetrazolium solution (Sigma-Aldrich). Simultaneously western blot analysis for Tubulin was also performed using its monoclonal antibody (Sigma Aldrich), which served as a control for equal loading.

2.7 Transient Transfections

Cultured cells were plated into six-well plates one day prior to transfection. The next day, cells were transfected using Transfast Reagent (Promega) as per the manufacturer's protocol. Forty-eight hours after transfection, the cells were harvested, lysed and assayed for luciferase activities as described earlier[14]. Plasmid encoding renilla luciferase was simultaneously transfected to normalize for transfection efficiencies.

3. Results

3.1 Cloning and sequencing of the AAAT repeat region in the *CTSL* gene.

The AAAT repeat containing region in the intron 1 (promoter) of *CTSL* was PCR amplified using the strategy shown in figure 1A. A 1.2 kb region was first amplified using a primary PCR with genomic DNA as template. This product served as a template for secondary and tertiary PCRs using internal nested primers. Thus, a 350 bp fragment of *CTSL* gene containing the repeats was amplified from the genomic DNA of different cell lines (Figures 1B, C & D). These were then cloned into pGEMT Easy vector (Promega Corp., USA) and sequenced (Microsynth, Switzerland). Sequencing of these clones revealed that in all the cell lines we analyzed, either 8 or 10 copies of the repeats were present (Figure 1E). Whereas the ten copy repeats were only restricted to neuronal cell lines (U87 and U373), we found that the eight copy repeats were more predominant (SiHa, Chang liver, HepG2, Hs294-T & H520). Hence, we observed variation in the lengths of "AAAT" repeats in *CTSL* promoter in cancer cell lines of different tissue origins.

Figure 1A: Strategy for amplifying CTSL AAAT repeats from genomic DNA

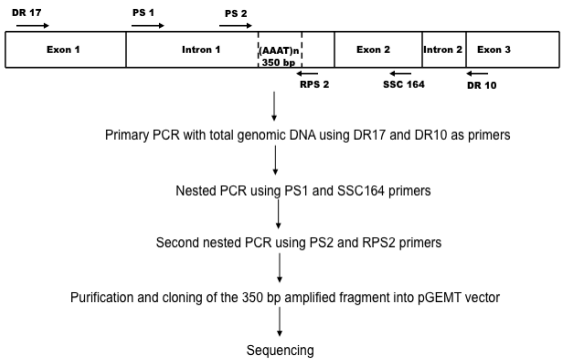

Fig 1A. The strategy used for amplifying the 350 bp AAAT repeat region in intron 1 is depicted here. A primary PCR was performed using genomic DNA as template. Subsequently, two nested PCRs were performed using the previous PCR products as template using internal primers as shown.

Figure 1B: Primary PCR for amplifying AAAT repeats

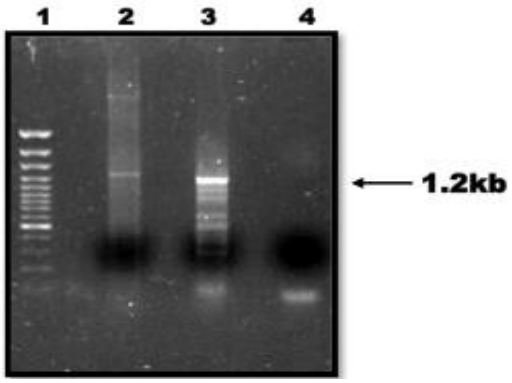

Fig 1B. Primary PCR was performed with genomic DNA as template using the primers DR-17 (Exon 1) and DR-10 (Exon 3). A 1.2 kb fragment was thus amplified containing a region of intron 1 and 2. Lane-1:- 100 bp ladder, Lane-2:- positive control plasmid (pGEMEP-1), Lane-3:- Genomic DNA (U87), Lane-4:- Negative control.

Figure 1C. Secondary PCR for amplifying AAAT repeats

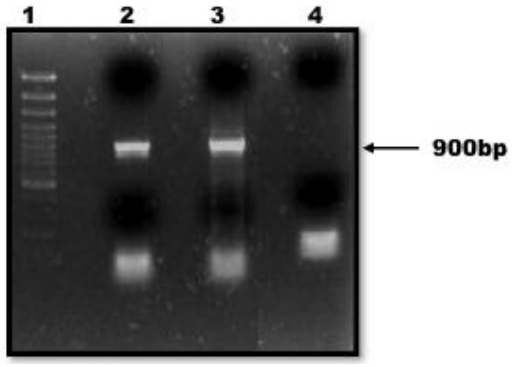

Fig 1C: Secondary PCR was performed with primary PCR product using internal primers, PS1 and SSC 164, from intron 1. A 900 bp fragment was thus amplified. Lane 1:- 100 bp ladder, Lane 2:- positive control (pGEMEP-7), Lane 3:- Primary PCR product, Lane 4:- Negative control

Figure 1D: Tertiary PCR for amplifying AAAT repeats

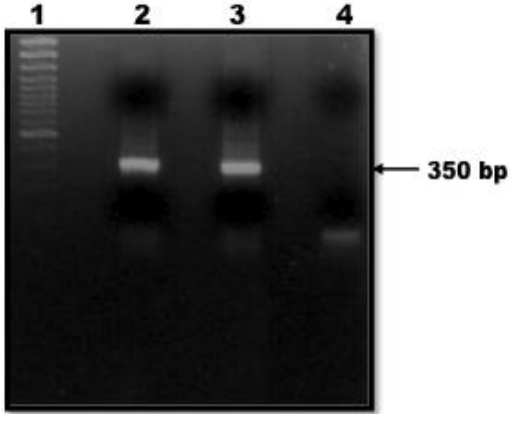

Fig 1D. A nested PCR was performed using secondary PCR product as the template. A 350 bp fragment of intron-1 containing the AAAT repeat region was thus amplified using PS2 and RPS2 as the primers. Lane 1:- 100 bp ladder, Lane 2:- positive control plasmid (pGEMEP-7), Lane3:- Secondary PCR product, Lane 4:- Negative control.

Figure 1E: Amplified and sequenced AAAT repeats

GGCAGAGGTTGCTGTGAGCCGATATCGCGCCGTTGAACTCCAGCCTGGGCCACAGAGCAAGACTCCATCT
C AAA**AAATAAATAAATAAATAAATAAATAAATAAATAAATAAAT**AGGAGAGATTGGAAAACTTATCT
CAGCTTTTGGTGTTTGTTAGTCAGGAAGATGTGTGAAGGCCTCCTAACTCTTGGGGATCTCTTTGTCCCT

Fig 1E. The sequence from the first intron of U87MG genomic DNA containing ten AAAT repeats (in bold) is shown here. We have found sequences with either eight or ten repeats in this region from different cell lines.

3.2. Length of the repeats directly correlates with CTSL expression levels.

To investigate if the varying length of AAAT repeats can differentially regulate CTSL expression, we compared the CTSL mRNA and protein levels in the various cell lines. A comparison of the total CTSL mRNA level in the above mentioned cell lines by real time PCR revealed that they were significantly higher in U87MG (32 fold) and U373 cells (5.4 fold) (Figure 2A). Both of these cell lines contained ten repeats and exhibited significantly higher CTSL mRNA levels as compared to those with eight repeats.

Figure 2A: CTSL mRNA levels in various cell lines

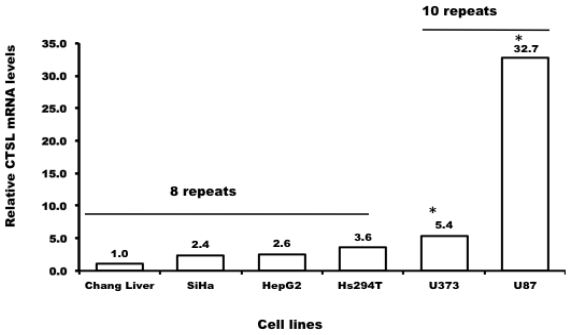

Fig 2A. 5 µg of total RNA from the cell lines was reverse transcribed and real time PCR was performed as described in materials and methods. Relative CTSL mRNA levels are shown as fold increase over Chang liver cells (taken as 1). Values are mean ± SD from at least three independent experiments. '*' - Indicates values significantly different [P<0.05] from Chang liver cells.

The CTSL protein was determined using enzymatic assays and western blotting. A fluorogenic synthetic substrate was used to detect the CTSL enzyme activity by spectroflourimetry as described in materials and methods. Again, the cell lines with ten repeats exhibited significantly higher CTSL activity (8.5 fold in case of U87 and 6 fold in case of U373) as compared to SiHa, which exhibited the lowest activity amongst those with eight repeats (Figure 2B). Also, no significant difference was observed among the cell lines with eight repeats. In addition, western blotting was also performed to compare the total CTSL protein levels from four of the representative cell lines (Figure 2C). The 'prepro' form of CTSL was only seen in U87 cells, which exhibited the maximum total CTSL expression. However, we did not observe any significant difference between U373 and other eight repeat containing cell lines used here. Since data from both the enzyme assays and real time PCR suggested that the ten repeat cell lines contained higher CTSL levels, we explored the possibility of the repeat lengths to differentially regulate CTSL levels in them.

Figure 2B: CTSL enzyme activity in various cell lines

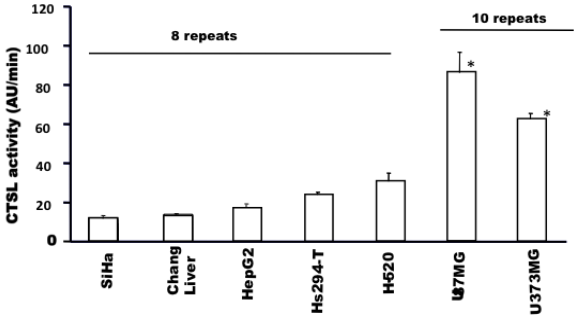

Fig 2B. CTSL enzyme activity was determined using 5µM fluorogenic substrate, CBZ-Phe-Arg-Nmec, as described in materials and methods. Specific CTSL activity was expressed as arbitrary units per µg of total protein. Values are mean ± SE from at least three independent experiments. '*' - Indicative of values significantly different [P<0.05] from those in cells with eight repeats.

Figure 2C: CTSL protein expression in various cell lines

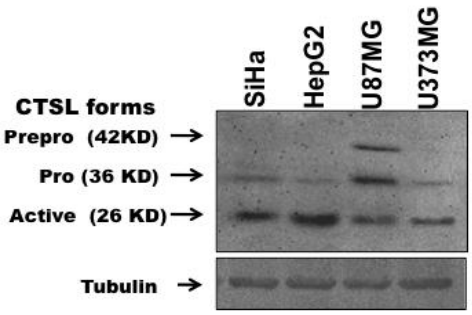

Fig 2C. CTSL protein was quantitated in the cell lines by western blotting using its monoclonal antibody. A representative picture from two of the eight repeat containing cell lines (SiHa, HepG2) and ten repeat containing cell lines (U87MG, U373) is shown. The prepro, pro and mature forms of CTSL were observed at their appropriate sizes. Tubulin protein was also similarly quantitated and served as an internal control.

3.3. Higher AAAT repeats up regulate heterologous transcription

To analyze the effect of varying lengths of AAAT repeats on transcription, they were cloned upstream to a T_k minimal promoter, which controlled firefly luciferase transcription. Hence, two constructs were generated, containing either 8 or 10 AAAT repeats upstream to the minimal promoter, labeled as pT_kL-8 and pT_kL-10. A similar vector with no repeats served as a control (pT_kL-0). It is noteworthy that since the T_k promoter here was a minimal one, it could result in significant luciferase expression only in the presence of a nearby enhancer sequence. The three T_k constructs (pT_kL-0, pT_kL-8 and pT_kL-10) were transfected into U87 cells (10 repeats) and HepG2 cells (8 repeats) and a comparison of the luciferase activities generated is shown in figure 3. Both the constructs with eight and ten repeats resulted in significantly higher luciferase expression than the one with zero repeats in both the cell lines. Since the repeat region significantly enhanced luciferase expression through a heterologous minimal promoter, it may contain transcriptional enhancer element/s. In HepG2 cells, we observed significantly higher expression from the pT_k-10 construct as compared to pT_k-8, suggesting that the variation in the repeat length can contribute to the observed differential CTSL levels. However, no such difference was observed upon similar transfections in U87 cells.

Figure 3: Variable repeats differentially regulate luciferase expression

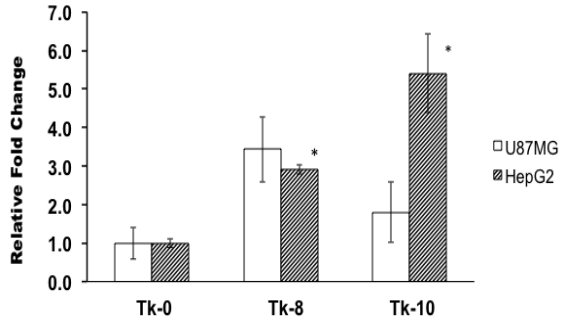

Figure 3: Recombinant plasmids containing either zero, eight or ten "AAAT" repeats cloned upstream to T_k minimal promoter (pT_kL-0, pT_kL-8 and pT_kL-10) were generated and transiently transfected into HepG2 and U87MG cells. The pT_kL-0 served as the control vector. The pRL null plasmid was also simultaneously transfected as a control for transfection efficiency. Firefly and Renilla luciferase activities were assayed 48 hrs after transfection. Normalized luciferase activities were plotted as fold increase over control vector. Values significantly different ($P<0.05$) compared to control vector are indicated by '*'.

4. Discussion

Cathepsin L is involved in a variety of physiological and pathological processes. Lots of studies are unraveling its role as a major determinant in cancer and metastasis[15][16][17]. Inhibiting CTSL decreases angiogenesis, results in reduced tumor growth and invasiveness[18][19][20]. Clearly, understanding the molecular mechanisms by which CTSL expression is regulated in cancer cells is important.

Two mRNA transcripts of CTSL, hCATL A and hCATL B, were independently cloned from SV40 transformed human fibroblasts[21] and human kidney library[22], respectively. Cloning and characterization of human *CTSL* gene revealed that these two mRNA species having identical coding region, but different 5' UTRs, were transcribed from the same gene located on chromosome 9q21-22.[9] It was also demonstrated that the 5'UTR of hCATL B is present in the 3' end of the first intron of hCATL A, contiguous with the second exon[14]. More recent studies have revealed that although the splice variants of hCATL A like AI, AII and AIII are concurrently expressed in different tumor cell lines, they are differentially translatable in them[10]. Our laboratory had for the first time cloned and characterized the two CTSL promoters, which generate hCATL A and B transcripts[14][23]. The promoter for hCATL B is a TATA-less promoter located in the first intron of the gene. This was found to harbor a number of "AAAT" repeats which when deleted, led to significant reduction in the promoter activity. This region contains multiple binding sites for potential regulatory motifs of Hepatocyte Nuclear Factor (HNF) family, which may play a role in the initiation of transcription in the absence of a TATA box.

Numerous reports suggesting the involvement of tandem repeat sequences in regulating gene expression are available[24][25][26]. A polymorphic tetranucleotide repeat consisting of $(AAAT)_n$ within the first intron of the parathyroid hormone gene has been used to investigate the segregation of the PTH gene[27]. Alu-repeat polymorphic sequence (AAAT) is also reported in intron 27 of neurofibromatosis gene (NF1) of neurofibromatosis patients[28]. Genetic polymorphism of the Aromatase (CYP19) gene intron 4 $(TTTA)_n$ was found to be associated with the familial prostate cancer risk[29]. Hogveen *et al*[2001] demonstrated that the activity of human sex hormone binding globulin (SHBG) promoter is influenced by a $(TAAAA)_n$ repeat element located at the 5' boundary of SHBG promoter. Polymorphic AAAT repeats have also been reported to influence diseases like Autism

and mental retardation[30]. Similar AAAT repeat polymorphisms have also been demonstrated to play a regulatory role in the expression of inducible nitric oxide synthase gene and protective role against the cognitive dysfunction[31]. Interestingly, we also observed the presence of such repeat sequences in the intron 1 of CTSL, which forms its promoter region. Also, earlier while we had performed promoter analysis, we observed a substantial decrease in its promoter activity upon deletion of the repeat region. Hence, in this study we have further investigated the role of these repeats in regulating CTSL expression.

Cloning and sequence analysis of the AAAT repeat region in CTSL promoter from cell lines of different tissue origins revealed that the number of these repeats were variable. We observed the presence of either eight or ten repeats in the cell lines tested. Out of the seven cell lines, only two (U87 and U373) contained ten repeats and the rest five (HepG2, Chang liver, SiHa, Hs292-T, H520) had eight repeats. Both the cell lines with ten repeats were of neuronal origin, whereas the others were of varied tissue origin. We further compared the CTSL expression levels in all the cell lines and correlated it with the number of repeats in these cells. CTSL protein levels were quantitated both by enzymatic assays and western blotting. CTSL enzyme activity was assayed by a previously established very sensitive spectrofluoremetric method using a synthetic substrate, CBZ-Phe-Arg-Nmec[14]. Interestingly, our results established that the CTSL enzyme and protein levels were significantly higher in cells with ten repeats as compared to those with eight repeats. Similarly, CTSL mRNA was also found to be more abundant in the ten repeat containing cells (32 fold in U87 and 5.4 fold in U373 as compared to Chang liver cells). The huge difference in RNA levels between U87 MG and U373 cell lines can be explained by their respective p53 status (wild type and mutant), as it has been shown by us that wild type p53 in U87MG cells causes CTSL transcriptional upregulation[32].

Thus, we observed a direct correlation between the length of repeats and CTSL expression levels, with the ten repeat containing cells exhibiting higher CTSL mRNA and protein levels. Since these repeats are in the promoter region of CTSL, we further investigated their role in transcriptional regulation. For this, transient transfections were performed with recombinant vectors containing eight or ten repeats cloned into the T_k Luciferase vector, which contained firefly luciferase cDNA driven by a T_k minimal promoter. A control vector with no repeats was also used for comparison. These vectors were then transiently transfected into HepG2 and U87MG cells and their luciferase readings compared after normalization. Eight and ten repeats resulted in significantly higher luciferase activities as compared to zero repeat in both the cell lines. Thus, both lengths of the repeats were by themselves able to induce expression from a heterologous minimal promoter. This demonstrated that the effect of repeats was not promoter specific, thereby suggesting them to contain enhancer element/s. Also, we observed significantly higher luciferase expression from pT_k-10 than pT_k-8 in HepG2 cells. However we did not observe any significant variation between 8 and 10 repeat constructs in U87 cells. The exact reasons for this are not known. We speculate that the endogenous ten AAAT repeats in this cell line competes with the transfected pTk-10 (10 repeats) plasmid for the transcription factors in the backdrop of already existing very high CTSL expression, resulting in no significant difference.

In conclusion, we have shown the presence of variable number of AAAT repeats in the CTSL promoter and demonstrated that the number of these repeats correlates with its expression levels in a number of cancer cell lines. In addition, we demonstrate that these repeats could significantly up regulate transcription from a heterologous promoter and hence may contain enhancer activity. We also observed that the ten AAAT repeats induced significantly higher transcription in case of HepG2 cells. This warrants further work on exploring the role of these repeats in differentially regulating CTSL expression in hepatocellular carcinoma patients.

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