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Immunotherapeutic potential of DISC-HSV and OX40L in cancer

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Abbreviations:-DISC-HSV (disabled infectious single cycle-herpes simplex virus), mGM-CSF (murine Granulocyte Macrophage colony stimulating factor), Gr1-Granulocyte Marker, CTL-Cytotoxic T lymphocytes, APC-Antigen Presenting Cells, (MTA1) Metastasis Associated Antigen 1

Abstract

Several vectors, viral and bacterial, have been developed over the past few years for means of generating an effective anti-tumor immune response. We have developed and studied a "model for immunotherapy" using a viral vector DISC-HSV, which efficiently transduces various tumor cell lines and offers a useful vehicle for the further development of cell based vaccines. The immunotherapeutic potential of DISC-HSV encoding GMCSF was demonstrated in a number of murine carcinoma models, leading to complete regression of well established tumors in up to 70% of the mice. Moreover, the therapeutic potential of DISC-HSV-GMCSF was significantly enhanced when used in combination therapy with either OX40L or dendritic cells (DC), even in poorly immunogenic tumor model. The ability of this vector to accept large gene inserts, its good safety profile, its ability to undergo only a single round of infection, the inherent viral immunostimulatory properties and its ability to infect various tumor cell lines efficiently, make DISC-HSV an ideal candidate vector for immunotherapy. The DISC- CT-26 tumor model has been used to investigate these mechanisms associated with immunotherapy – induced tumor rejection. Although CTL induction, was positively correlated with regression, MHC class I down regulation and accumulation of immature Gr1+ myeloid cells were shown to be the main immuno-suppressor mechanisms operating against regression and associated with progressive tumor growth. The CTL response has been associated with the immuno-dominant AH-1 peptide of the retroviral glycoprotein gp70. This model of immunotherapy has provided an opportunity to dissect further the immunological events associated with tumor- rejection and escape. Since other antigens may be important in initiating tumor rejection, we have investigated the expression of MTA-1, an antigen that appears to be expressed widely in human and murine tumors. The immunogenicity of MTA-1 has been studied and its potential as a tumor rejection antigen is under investigation.

General Introduction:

The relatively recent identification of human and murine tumor antigens has led to a resurgence of interest in immunotherapy, previously directed at the activation of antigen – specific T-cells [9,28,32]. It is therefore important to gain insight into the contributing factors governing rejection verses tumor escape from both human and model systems. A limited number of models exist which allows detailed analysis of the immune systems in mice exhibiting progressing or regressing tumors following immunotherapy. In this review we describe the use of a potent viral vector, Disabled Infectious Single Cycle-Herpes Simplex Virus (DISC-HSV), as a useful vehicle for the efficient transduction of cytokine genes into tumor cells and capable of promoting immunotherapy against established tumors. The DISC-HSV/CT26 tumor model has proved to be useful for investigating immune escape mechanisms. The immune response in this model was correlated with CTL responses against an endogenous murine leukemia virus antigen gp70 and the following summarises our recent finding that suggests possible pathways for immune escape and the identity of additional cancer associated target proteins.

DISC-HSV as a vector for immunotherapy

Over the past few years a number of viral vectors have been investigated not only for their ability to transfect autologous or allogeneic cell lines for cell based vaccines but also for directly delivering the tumor antigens to antigen presenting cells (APC) *in vivo* in an effort to generate an immune response. An ideal vector should be able to infect a broad range of cell lines efficiently, have a very good safety profile, should not be highly immunogenic itself, should be able to accept large cDNA inserts and should be able to infect dividing and non-dividing cells [8]. DISC-HSV is a herpes simplex virus (HSV) in which the gene for glycoprotein H (gH) has been deleted and hence this virus is only capable of a single round of infection. This property allows the virus to propagate after infecting the susceptible cells but the progeny are non-infectious, thus preventing the subsequent infection of other cells [13]. Moreover, HSV virus has been very well characterized with extensive safety profile in human clinical trials [23,50]. Also, DISC-

HSV has a 150 kb genome, which makes it convenient vector for the insertion of large amounts, of approximately 30kb, of genetic material [8].

Genes encoding a variety of cytokine or co-stimulatory molecules can be inserted into DISC-HSV, and subsequently used to infect cell lines and tissues, of both murine and human origin [51,62]. The transduction efficiency of this virus was found to be comparable to adenovirus. A panel of tumor cell lines were efficiently infected with DISC-HSV encoding the gene for granulocyte macrophage colony stimulating factor (GM-CSF) and GM-CSF secretion was observed continually up to 72 hours post infection [62]. Moreover, it was observed that DISC-HSV infection of tumor cells inhibited cell growth and induced necrotic, rather than apoptotic cell death; thereby providing the immune system with the essential 'danger signals' [37,62]. Moreover, herpes infections are known to cause inflammation that could activate the APC, and induce the up regulation of IL-12 expression, [25,41].

Considering the *in vitro* efficiency of DISC-HSV to infect the cells, its *in vivo* potential for treatment of established tumors has been studied. DISC-HSV encoding mGM-CSF, when injected intra-tumorally induced the complete regression in up to 70% of the animals and immunity to further challenge with the parental tumors [3,62]. These results were confirmed in other tumor models including renal and breast carcinoma [4,34,48,62]. Interestingly, "empty" DISC-HSV, or virus expressing an irrelevant gene could also generate an anti-tumor immune response [62]. This therapeutic effect could be, due to the inherent immuno-stimulatory properties of the virus and necrotic death of the infected cells, and subsequent activation of dendritic cells. Interestingly intra-tumor administration of DC's together with DISC-HSV–m GMCSF proved more effective than virus alone.

The observation that, DISC-HSV, when combined with dendritic cells (DC) in a combination therapy, caused regression in up to 100% of the animals signifies the role of the antigen presenting cells in development of the immune response against tumors [3]. The immune rejection of tumors treated with DISC-HSV based immunotherapy was

mediated by CD8+ cytotoxic T lymphocytes (CTLs), thus no tumor regression or protection was observed in Balb/c nude mice or on depletion of CD8+ T cells [3].

One of the major concerns of immunotherapy using a viral vector is the presence of pre-existing immunity to the vector, which might limit the efficacy of the vaccine. However, experimentally previous exposure to HSV-1 virus did not limit the therapeutic efficacy of the DISC-HSV therapy in the CT26 tumor model [3]. Moreover, the systemic immunity developed in these tumor models in response to DISC-HSV therapy prevented the tumor growth at distant sites. This systemic immunity to CT26 tumors was observed to be mediated by CTLs directed mainly against the AH-1 peptide of the gp70 tumor antigen, which is naturally processed by the CT-26 tumor cells. CD4+ T cells were shown to be an essential component of tumor rejection, as their depletion decreased the level of anti-tumor immunity. Adoptive transfer experiments using AH-1 peptide specific CTLs into tumor bearing animals led to regression of tumors in these animals whereas administration of CTLs directed against an irrelevant peptide did not influence tumor growth. Not only was the presence of these CTLs observed in the tumors by immunohistochemistry, but their specific trafficking into the tumors was also confirmed by real time *in vivo* microscopy (IVM) using fluorescently labeled CTLs [4,5].

In conclusion, DISC-HSV could not only prove to be an important vector for delivery of cytokine genes into tumor cells but could act as an immune adjuvant capable of presenting tumor specific immune responses. Moreover, pre-existing immunity to HSV does not affect the efficacy of DISC-HSV therapy, which has also been reported to have an excellent safety profile as shown in clinical trials for primary and recurrent HSV-2 [23].

DISC-HSV and OX40L as a combination Therapy

Optimal activation and development of naïve T cells into effector and/or memory cells requires two signals. The first signal is provided by the binding of T cell receptor with MHC-peptide complex on the APC, whereas the second co-stimulatory signal is

generated by interaction between molecules such as CD40-CD40L, CD28-CD80/CD86 and CD27-CD70. This co-stimulatory signal is essential for an effective immune response development, as its absence can lead to anergy and apoptosis of antigen specific T cells. Interaction of CD28 with its ligand occurs early during this process and leads to up regulation of other co-stimulatory molecules such as OX40 and 4-1BBL.

OX40 (CD134) is a member of the TNF receptor superfamily. OX40 is an interesting and unique molecule to target in cancer immunotherapy as it is only expressed on activated T cells. OX40 ligation leads to clonal expansion and proliferation of the activated T cells and OX40 expression peaks on the activated T cells after approximately 48 hours [57]. OX40 ligand is expressed on the APCs and OX40 ligation, has been shown to be essential for the generation of the memory T cells and their survival. More studies in OX40 / OX40L knock out mice clearly show an impaired CD4$^+$T-cell as well as defective dendritic cell mediated cytokine production and co-stimulation [12].

 One of the main aims of cancer immunotherapy is to generate memory T cell responses in order to prevent relapse of the disease after initial clearance of the tumor. OX40 molecules seem to be an ideal target to achieve this aim. Indeed, OX40 therapy in tumor bearing animals, through agonist antibodies or OX40L-immunoglobulin fusion proteins, enhances anti-tumor immune response and increased tumor free survival in various tumor models [6,27,40]. It has been speculated that OX40 mediated stimulation helps in the generation and survival of CD4+ T cells with a memory phenotype and provides help to generate effector CTLs [38]. Also, these CD4+ T cells might have a more direct role in tumor cell killing as reported by various studies [15,49]. Tumor infiltrating CD8+ T cells have also been observed to express OX40 molecules, which suggest that OX40 might directly or indirectly enhance CTL function [26,57].

As OX40 is mainly involved in proliferation of activated T cells and the generation of a memory phenotype, it could serve as an agent of value in promoting tumor antigen specific responses when used in a combination immuno therapy. OX40L was recently used in a combination therapy with another co-stimulatory molecule anti-4-1BB and

adenovirus encoding for IL-12, which led to regression of large established hepatic metastasis. Interestingly, this model required the presence of all three agents to achieve maximum benefit, whereas eliminating any of the three agents from the combination severely compromised the efficacy of the therapy [47]. Another study demonstrated that C26 carcinoma cells co-transfected with GM-CSF and OX-40L were rejected by 85% of the animals, whereas injection of either GM-CSF or OX-40L transfectants alone only achieved a delay in tumor onset [19]. The advantage of using OX40L for combination cancer immunotherapy was clearly demonstrated in both studies. Moreover, in both these models, CD4+ T cells were essential and long term memory was established as immunized animals rejected the challenge of parental tumor cells after therapy. However, none of the studies investigated the possible effector functions of the CD4+ T cells. The therapeutic efficacy of OX40 mediated therapy is dependant on the anatomic site of the tumor growth, tumor burden and the intrinsic immunogenicity of the tumor [26].

The potential of OX40L and OX40L/DISC-HSV combination therapy in inducing the regression of established tumors is shown in figure 1 and has been previously reported [6]. In two histologically different tumors, it was observed that intra peritoneal injection of mOX40L-immunoglobulin fusion protein led to a significantly reduced tumor growth in tumor bearing animals and up to 50% of animals remained tumor free for the duration of the experiments, whereas no protection was observed in animals given control therapy in the form of either PBS or human OX40L. This protection was dependant on the timing of the therapy, the dose and the route of administration of OX40L. Five doses of 250μg mOX40L given intra-muscularly significantly improved the response. Moreover, timing was crucial in generating the maximal benefit of OX40L therapy, as OX40L administration three days after tumor cell inoculation protected 7 out of 10 mice whereas significantly less protection was observed, when the therapy was started before or after three days [6]. This may be due to the transient expression of OX40 on the activated T cells, which peaks at approximately 3 days coinciding with the maximal activation of these T cells [56]. Moreover, OX40L also reduced the development of the experimental metastases in 50% of the animals that remained tumor free for the duration of the experiment. Combined OX40L and DISC-HSV therapy in a moderately immunogenic

4T1 tumor model, inhibited tumor growth rate and caused tumor regression in up to 70% of animals, which were immune to further challenge by the parental tumor cells. Furthermore, in a poorly immunogenic tumor model, RENCA, DISC-HSV on its own provides little if any therapeutic benefit; but when combined with OX40L, tumor regression was observed in approximately 40% of the animals. Investigating the tumor rejection mechanism in CT-26 tumor model confirmed the involvement of both CD4+ and CD8+ T cells, as therapy proved ineffective in CD4+ or CD8+ T cell depleted mice. This again suggests that $CD8^+$ CTLs mediate the effector function but require CD4+ T cells for optimal activation. Additionally, CD4+ T cells might have a direct anti-tumor role since the depletion of CD8+ T cells does not abrogate the response completely [6].

Immuno Escape mechanisms in DISC-HSV/CT26 model

Cancerous cells are genetically unstable, and may change their phenotypic characteristics in order to evade the immune system. In fact, it has been suggested that the immune system helps tumor cells to develop a more aggressive phenotype by eliminating the more vulnerable cells from a heterogeneous population, a phenomenon termed as 'immunoediting' [14]. Some mechanisms employed by tumors in order to evade immune attack are: down regulation of their MHC molecule expression, loss of the expression of an immuno-dominant antigen, release of immuno-suppressive cytokines such as TGF-β and IL-10, expression of FasL that may induce apoptosis of activated T cells and the activation of the immuno-regulatory cells such as Natural Killer T (NKT) cells and CD4+CD25+ T Regulatory cells [1,11,22,60]. Hence, immune response generated against a tumor may only lead to temporary regression, unless it is accompanied by a strategy to counteract immuno-escape mechanisms. It is clear from research in this field that different tumors employ different mechanisms to evade the immune system.

As discussed previously, DISC-HSV therapy in CT-26 bearing animals leads to tumor regression in a high proportion of animals, but approximately 40% of treated mice will continue to progress their tumors [3,62]. This model has allowed the study of immune-escape mechanisms employed by the progressor mice and develop strategies to prevent them. It was observed that the majority of progressor tumors from mice receiving DISC-

HSV therapy had low (55%) or no (45%) expression of the MHC class I molecules whereas only 50% of tumor bearer animals (not receiving therapy) had partial down regulation of MHC antigens, the remaining 50% showing no loss of MHC class I expression [1]. Interestingly, the tumor cells isolated from progressor or tumor bearer mice up-regulated their MHC class I expression following overnight culture *in vitro*, showing this phenomenon to be reversible and demonstrating the dynamic nature of phenotypic change associated with their growth environment.

Another potential mechanism of immune-escape associated with the CT26 model is the accumulation of immature Gr1+ myeloid cells. Gr1+ cells were significantly increased in the spleens and the blood of animals with progressive tumors, and their presence was also detected within the tumors of mice failing to respond to DISC-HSV or OX40L therapy [2]. Interestingly, these immature forms of myeloid Gr1+/CD11b+ cells have also been identified in other mouse models and cancer patients [7,10,17] and shown to inhibit the activation of the primary T cells [30]. However, the exact role of Gr1+ cells in suppressing the immune response to tumors is not yet known. Immature myeloid Gr1+/CD11b+ cells can be induced to differentiate into mature DC through intratumoral gene delivery of GM-CSF leading to activation of the natural killer cells and CTLs in mice treated with combination therapy of IL-12 and anti 4-1BB [33]. Another study has recently shown that administration of all trans retinoic acid (ATRA) along with antigen specific vaccination could induce differentiation of immature myeloid Gr1+ cells into mature DCs improving the functional ability of anti-tumor CD4+ and CD8+ T cells and leading to objective regression of large tumors [31]. Tumor inhibition was also enhanced by co-administration of retinoic acid (ATRA) with DISC-mGMCSF therapy in CT26 tumor model (Ali et al, unpublished observation).

NKT cells have recently generated substantial interest as CD4+ or double negative (CD4-CD8-) sub-populations of T cells, which also express NK cell markers, can produce large amounts of cytokines, especially IL-13 and act as immunosuppressive cells [18,39,55]. However, some studies indicate that NKT cells might be important in anti-tumor immunity [55,58]. Recently, a model of negative regulation of immune response against

cancer was proposed by Terabe et al, briefly: the NKT cells upon activation by glycolipid antigen on APCs secrete IL-13, which acts on the Gr1+/CD11b+ cells, inducing them to secrete TGF-β which suppresses antigen specific CTL responses [61]. Initial studies in the DISC-HSV/CT26 model failed to confirm the involvement of NKT cells or IL-13 production from CD4+ cells isolated from progressor mice splenocytes. However, CD4+ T cells isolated from the splenic parenchymal tissue of mice failing to respond to DISC-HSV therapy can be immunosuppressive and shown to inhibit antigen specific CTL activity [Ahmad et al, in press]. Although these T cells were CD4+CD25-, initial results suggest that they are not NKT cells as no significant difference in IL-13 production was observed.

Limitations of the DISC-HSV/CT26 model

Although, the CT-26 model allows us to study the anti-tumor associated immune responses and the escape mechanisms, it has certain limitations. Firstly, the CT26 murine colon carcinoma has been established by sub-cutaneous implantation and tumors develop rapidly, whereas most human cancers grow slowly over a period of years, allowing multiple mutations to accumulate and the likely development of "immune escape" tumor variants. Also, the immuno-dominant epitope in the CT-26 cell line is derived from murine leukemia virus gp70 whereas most tumor antigens are over-expressed or mutated 'self antigens'. However, recently it has been shown that certain human cancers express endogenous retroviral sequences although it is not known as to whether they are associated with malignant transformation [16,43].

MTA1-a potentially novel metastasis associated antigen for immunotherapy

In a search for "novel" cancer-associated antigens that are broadly expressed in cancers, Metastasis Associated Tumor antigen 1 (MTA1) can be proposed as a suitable candidate. Originally identified using a differential cDNA library screening using rat mammary adenocarcinoma cells [63] the MTA1 gene encodes an 82 kDa protein. Analysis of the gene sequence of MTA1 revealed that it has a proline rich region (SH3 binding motif),

putative zinc finger DNA binding motif and a leucine zipper motif. Moreover, the human MTA1 protein is also rich in SPXX motifs, which are usually expressed in gene regulatory and DNA binding proteins. Also, it contains three nuclear localization signals [44,46]. Although consisting of motifs usually found in transcription factors, it was shown recently that MTA1 might be a part of the nucleosome remodeling (NuRD) and histone deacetylase complex (HDAC) and thus inhibit transcription [65]. Histone proteins help in the organization of the DNA into nucleosomes, which are regular repeating structures in the chromatin and the acetylation status of the histone proteins effect gene expression by altering the transcription of the genes [36]. Moreover, the expression level of MTA1 correlated inversely with the acetylation status of histone H4 in invasive oesophageal carcinomas, which correlated positively with the prognosis of the patients [66]. This evidence suggests that MTA1 might be localized in the nucleus and act to repress transcription [44].

Other members of MTA1 family have recently been identified, MTA2, MTA3 and MTA1s. MTA2 and MTA3 might possibly have similar functions to MTA1 in terms of histone modification and transcriptional regulation [54,68], whereas MTA1s is a naturally occurring shorter variant of MTA1, expressed in breast cancer tissues and is capable of sequesting oestrogen receptor-α in the cytoplasm, making these cells unresponsive to hormonal therapy [29].

Although MTA1 is expressed at very low levels in most tissues of the body, various studies have confirmed the association of MTA1 over-expression with the increased tumor growth and invasive potential; gastric and colorectal carcinomas over-expressing MTA1 showed significantly higher rates of invasion and lymph node metastasis [64]. In similar studies, DNA microarray analysis was used to investigate MTA1 expression in 300 patient samples comprising of metastatic prostrate cancer, clinically localized prostrate cancer and benign prostrate tissue, MTA1 expression was correlated positively with malignant potential [24]. Human laryngeal squamous cell carcinomas, lung cancers, ovarian carcinomas, hepatocellular carcinomas and breast cancers also demonstrate MTA1 to be associated with tumor aggression [21,45,53,59,69].

Recently it has been demonstrated that inhibition of MTA1 expression in MDA-MB-231 breast cancer cells by anti-sense phosphorothioate oligonucleotides resulted in inhibition of their growth and invasiveness [46]. Another recent study investigated the functional role of MTA1 expression in cancer cells [35]. MTA1 overexpression in an immortalized human keratinocyte cell line increased its migratory and invasive potential, allowed cell survival in the anchorage independent state and also led to increased expression of anti-apoptotic Bcl-2 family member Bcl-xl. Thus, MTA1 over expression might support various steps in the transformation process of normal cells [35].

Overexpressed cancer-associated proteins, particularly those involved in cell transformation represent potential targets for immunotherapy. Considering the expression pattern and the diversity of MTA1 expression in different tumors, MTA1 might serve as a target for immunotherapy. MTA1 is expressed in normal tissues at lower levels and hence there is a risk of inducing auto-immunity. We have confirmed the low level expression of MTA1 in various normal murine tissues compared to very high levels of expression in almost all murine tumor cells lines that were tested (Figure 2). However, similar self-antigens, for example like p53 and survivin, have been used in vaccines without severe adverse effects [42,52,67].

MTA1 has been identified as a SEREX antigen and hence it is likely to be capable of inducing a T-cell response in cancer patients [32]. The protein is highly conserved between mouse and man, 86% and 96% identity of the nucleotide and protein sequences respectively, and appears to perform similar functions in both species. This provides an opportunity to establish models for investigating MTA1 immunotherapy where initial studies have demonstrated the presence of immunogenic MHC class I restricted peptides (Assudani et al unpublished observations). In a search for peptides it is important to consider that to CTL repertoire against high affinity binding peptides is partially tolerated, which may not be the case for moderate to low affinity peptides [20].

Conclusions and Future Directions

DISC-HSV could be a potential vector, not only for *in vitro* transduction of cells but also as a vector for the *in-vivo* delivery of "immune response" genes or antigens. Therapy with DISC-HSV is safe and is not affected by previous exposure to HSV infection. Regression of established tumors in approximately two-thirds of animals receiving DISC-HSV immunotherapy can lead to the generation of tumor specific immunity.

In combination with cell-based or cytokine immuntherapies DISC-HSV can promote high levels of CTL effectors and immunity to tumor antigens. It will be important to design future immunotherapy strategies to target antigens, such as MTA1, that are correlates of tumor aggression.
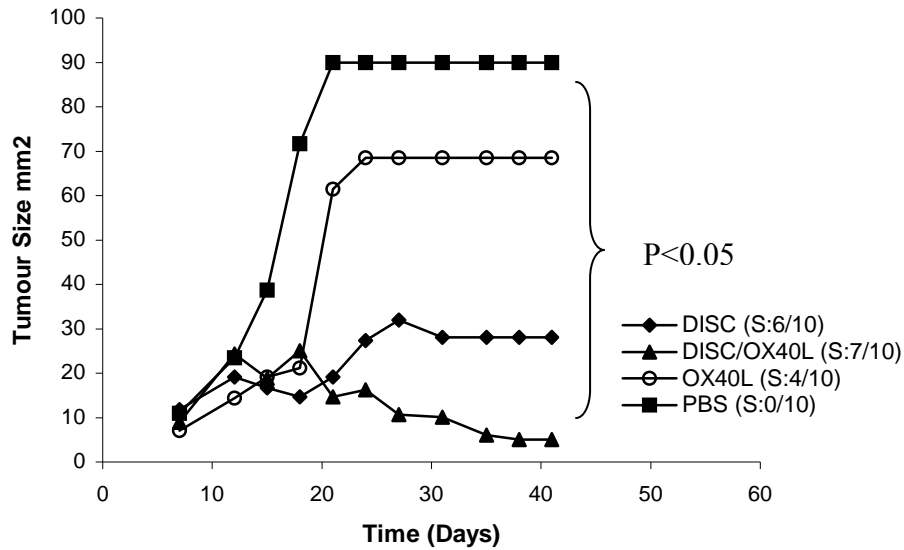
Figures

Figure 1:- Combination therapy of DISC-HSV/mGMCSF and OX40L in a CT-26 tumor model. Four groups of 10 BALB/c mice were implanted s.c. with 1×10^4 CT26 cells. Therapy was initiated when tumors reached the size of 0.09–0.36 cm^2, which is usually on days 8–10. DISC: micewere injected twice at 2 days intervals with 2.5×10^7 pfu DISC/mGM-CSF virus. OX40L: mice were injected i.p. with 250 _g per mouse on days 8and 14. DISC + OX40L: mice were first injected intra-tumorally with DISC virus and concomitantly injected i.p. with OX40L fusion protein as above.PBS: mice were injected intra-tumorally and i.p. with PBS as control. (Adapted from Ali et al, 2004)
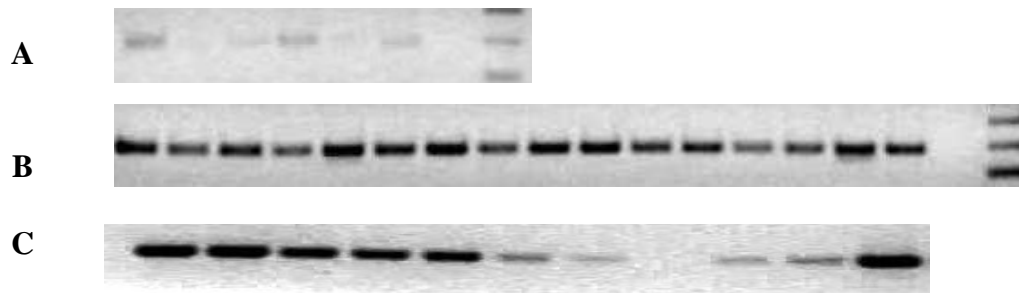
Figure 2:- Determination and comparison of expression of MTA1 in normal human tissues, human tumor cell lines, murine tissues and murine tumor cell lines using RT-PCR.

(A) From left to right, Normal human tissues, lanes 1-6= Brain, Heart, Lung, Trachea, Liver, Kidney

(B) From left to right, Human tumor cell lines, lanes 1-16, HT29, ISUL, T47D, BB49, SENY, FM487, Jurkat, MCF 7, MEL 270, T2, JY, GERL 4.3, FM3, DBTRG, 293, T98G

(C) From left to right, murine tumor cell lines and tissues, lanes 1-11, CT26, A20, Renca, CMT 93, B16, Liver, Lung, Muscle, Spleen, Kidne, Testis

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