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MDA-7/IL-24 as a cancer therapeutic: from bench to bedside

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Abstract

The novel cytokine melanoma differentiation associated gene-7 (*mda-7*) was identified by subtractive hybridization in the mid-1990s as a protein whose expression increased during the induction of terminal differentiation, and that was either not expressed or was present at low levels in tumor cells compared to non-transformed cells. Based on conserved structure, chromosomal location and cytokine-like properties, MDA-7, has now been classified as a member of the expanding interleukin (IL)-10 gene family and designated as MDA-7/IL-24. Multiple studies have demonstrated that expression of MDA-7/IL-24 in a wide variety of tumor cell types, but not in corresponding equivalent non-transformed cells, causes their growth arrest and ultimately cell death. In addition, MDA-7/IL-24 has been noted to be a radiosensitizing cytokine, which in part is due to the generation of reactive oxygen species (ROS) and ceramide that cause endoplasmic reticulum stress. Phase I clinical trial data has shown that a recombinant adenovirus expressing MDA-7/IL-24 (Ad.*mda-7* (INGN-241)) was safe and had measurable tumoricidal effects in over 40% of patients, which strongly argues that MDA-7/IL-24 may have significant therapeutic value. This review describes what is known about the impact of MDA-7/IL-24 on tumor cell biology and its potential therapeutic applications.

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Keywords

MDA-7: melanoma differentiation associated gene 7

The discovery of MDA-7/IL-24 and its biologic effects

MDA-7/IL-24 was discovered using a subtraction hybridization approach by exposing melanoma cells to the terminal differentiation –inducing agents interferon beta and mezerein [1–3]. Based on a conserved amino acid signature sequence, chromosomal location and cytokine-like properties, *mda-7*, has been classified as a member of the expanding interleukin (IL)-10 gene family, which includes IL-10, IL-19, IL-20, IL-22 and IL-26, and has been designated as *mda-7/IL-24* [3–8]. MDA-7/IL-24 protein expression is decreased in advanced melanomas, with nearly undetectable levels in metastatic disease, in general agreement with this gene product being classified as a tumor suppressor [3–5]. Other published studies over the last 15 years have demonstrated that enforced expression of MDA-7/IL-24, either by transfection of tumor cells with a plasmid containing the cDNA for *mda-7/IL-24* or by use of a recombinant adenovirus to deliver the gene, *Ad.mda-7*, rapidly inhibits the growth of a broad-spectrum of cancer cells, resulting in tumor cell death within 24–48 h [1–11]. When expressed, MDA-7/IL-24 is secreted from cells, as would be expected for a cytokine. Of considerable note, when MDA-7/IL-24 was over-expressed in non-transformed cells little change was observed in either cell growth or cell viability [1–12].

Initial studies using mammalian cell synthesized MDA-7/IL-24 protein; a protein that is a dimer and glycosylated, demonstrated that purified MDA-7/IL-24 interacted with two type II cytokine hetero-dimeric receptor complexes: IL-20R1/IL-20R2 (type 1 IL-20R) and IL-22R1/IL-20R2 (type 2 IL-20R) [13] (Figure 1). In one of the first of these studies, non-transformed BHK cells stably transfected with IL-20 and IL-22 receptors were treated with MDA-7/IL-24; at low pM concentrations of MDA-7/IL-24 (<100 pM) growth was promoted whereas at higher concentrations (>100 pM) it inhibited cell proliferation. In cells transfected to express IL-20 receptor complexes, MDA-7/IL-24 activated multiple STAT transcription factors. However, in ovarian carcinoma cells, which express endogenous IL-20 receptor complexes, it was noted that MDA-7/IL-24 at low nM concentrations promoted growth inhibition without altering STAT transcription factor phosphorylation/function [13,14]. Other studies have demonstrated using tumor cells, which lack STAT1 or STAT3 function or with blocked Janus kinase function that STAT pathway signaling is not required for MDA-7/IL-24-induced growth arrest or tumor cell killing [15]. These studies used both viral and protein delivery of MDA-7/IL-24 to show a lack of STAT factor involvement.

More recently, studies have indicated a difference in the cell signaling and cell killing properties between bacterial synthesized unglycosylated and monomeric GST-MDA-7/IL-24 and mammalian cell synthesized glycosylated dimeric MDA-7/IL-24 with FLAG or (His)₆ tags to aid its purification. In multiple studies using a wide variety of transformed cell lines, GST-MDA-7/IL-24 has been noted to promote cell growth arrest and apoptosis in a tumor cell-specific fashion and has been noted to cause these effects independently of expression of IL-20 receptor complexes, in a similar manner to intracellular delivery of MDA-7/IL-24 via infection with *Ad.mda-7* [16,17, and references therein]. This would suggest that GST-MDA-7/IL-24 is taken up by cancer cells in an interleukin receptor independent fashion. In contrast to GST-MDA-7/IL-24 and *Ad.mda-7*, purified MDA-7/IL-24, synthesized in mammalian cells, does not appear to have any biologic effect on cells lacking expression of IL-20 receptor complexes. Of note however, and in a similar manner to GST-MDA-7/IL-24

and Ad.*mda-7*, in cells where IL-20 receptor complexes were expressed, mammalian synthesized MDA-7/IL-24-induced cell killing was independent of STAT transcription factor activation.

For example, in A549 human lung carcinoma cells, which lack expression of the IL-20 receptor complexes, extracellular treatment with mammalian cell synthesized MDA-7/IL-24 results in no biologic effect on cell growth/viability. In contrast, treatment with GST-MDA-7/IL-24, or viral infection with Ad.*mda-7* or Ad.*mda-7* signal peptide null (SP-), which expresses a non-secreted form of MDA-7/IL-24, or transfection of these cells with a plasmid to express MDA-7/IL-24, results in tumor cell growth arrest and cell death [18,19]. Furthermore, whilst it has been noted that MDA-7/IL-24, IL-20, and IL-19 all activated STAT transcription factors in IL-20 receptor expressing cancer cells, only MDA-7/IL-24 has the ability to cause cell death [20]. Thus the biologic differences between GST-MDA-7/IL-24 and His6-MDA-7/IL-24 and adenoviral/plasmid delivery of MDA-7/IL-24 into a cell, particularly with respect to findings from the A549 model, is of *primary* importance in understanding the difference between MDA-7/IL-24 acting as a cytokine in the “classic” sense of Interleukin biology and MDA-7/IL-24 acting as a protein which causes a toxic ER stress response (see below). It has been the comparison between whether MDA-7/IL-24 was delivered internally or externally in a cell lacking the receptors for the cytokine that ultimately permitted the insight into how MDA-7/IL-24 entered cells; the separation of receptor mediated signaling from ER stress signaling; and developed a greater understanding of MDA-7/IL-24 as a secreted cytokine with a “toxic bystander effect.”

MDA-7/IL-24 and transformed cell killing

The pathways by which Ad.*mda-7* (or: transfection with a cDNA to express MDA-7/IL-24; treatment with bacterial synthesized GST-MDA-7/IL-24 or eukaryotic cell generated His6-MDA-7/IL-24) enhances apoptosis in tumor cells are still not completely understood, however, over the last 5 years a large amount of evidence from multiple studies using each of these tools has demonstrated the involvement of proteins important in the regulation of endoplasmic reticulum (ER) stress and mitochondrial integrity [21–26]. Some studies have argued that MDA-7/IL-24 promoted activation of the double stranded RNA –activated kinase, Protein Kinase R (PKR), which was correlated with enhanced eIF2 alpha phosphorylation and MDA-7/IL-24-stimulated cell death. In this study PKR null fibroblasts were resistant to IL-24-induced apoptosis, although subsequent studies from the same group have argued that PKR does not always play a role in the lethal effects of MDA-7/IL-24 [27,28].

In studies from our laboratories using GST-MDA-7 and Ad.*mda-7* we noted that MDA-7/IL-24 protein binds to the HSP70 family chaperone BiP/GRP78 (Figure 1). Binding of MDA-7/IL-24 to BiP/GRP78 inactivates the chaperone function of the protein promoting its dissociation from PERK-like endoplasmic reticulum kinase (PERK) [21]. Over-expression of BiP/GRP78 suppresses MDA-7/IL-24-induced toxicity [22,23]. Dissociation of BiP/GRP78 from PERK promotes PERK trans-phosphorylation and activation, and subsequently the phosphorylation and activation of eIF2 alpha (Figure 1). The phosphorylation of eIF2 alpha in turn leads to the global suppression of protein translation which, with respect to its tumor cell killing properties, results in reduced expression of anti-apoptotic proteins that have short half-lives such as MCL-1, BCL-XL and c-FLIP-s [29–31]. Indeed, some of the earliest correlative observations regarding MDA-7/IL-24 toxicity were that the cytokine decreased expression of BCL-XL and enhanced expression of toxic BH3 domain proteins such as BAX and BAK [32,33 and references therein]. Activation of eIF2 alpha also activates ATF4 which leads to increased levels of Growth Arrest and Differentiation and Death (GADD) transcription factors e.g. GADD153 (CHOP) and GADD34. Very recent data from our

laboratories has also demonstrated a central role for the PERK-dependent generation of the lipid second messenger species ceramide and dihydro-ceramide in response to MDA-7/IL-24 [23,34,35]. One mechanism by which MDA-7/IL-24 likely increases dihydro-ceramide levels in a PERK dependent fashion is via increasing ceramide synthase 6 protein stability (Figure 1). Elevated ceramide levels facilitate calcium ion –dependent generation of reactive oxygen species that together all play a central role in modulation of signaling pathway function (see below) and mitochondrial integrity.

What is perhaps more unusual with respect to the cancer therapeutic properties of MDA-7/IL-24 compared to multiple other FDA approved anti-cancer agents, and in a manner consistent with the phrase “water always wins” is that in all tumor and transformed cells tested to date, intracellular delivery of this cytokine protein causes cell death; however, the precise mode of cytokine lethality exhibits subtle differences between tumor cells of different tissue origins [36,37]. One difference between cell types is the degree to which different toxic BH3 domain proteins play as upstream agonists promoting mitochondrial dysfunction. For example, the ability of Ad.*mda-7* to induce apoptosis in the prostate cancer cell line, DU145, which does not produce BAX, indicates that MDA-7/IL-24 can mediate apoptosis in tumor cells by a BAX-independent pathway [38]. In multiple primary human glioblastoma cells we noted downstream of PERK activation and lysosomal dysfunction that cathepsin B–dependent cleavage of BID played a central role in cytokine–induced mitochondrial dysfunction and lethality [22–24]. In a cell type dependent fashion MDA-7/IL-24 inactivates the ERK1/2 and activates the JNK signaling pathways leading to: dephosphorylation of BAD S112 and BIM, which promotes BAD activation and BIM protein stabilization and activation of BAX and BAK, respectively. In melanoma cell lines, but not in normal melanocytes, infected by Ad.*mda-7* or treated with GST-MDA-7, it was noted that a significant decrease in both BCL-2 and BCL-XL levels occurred, with a more modest up-regulation of BAX and BAK expression [39]. This data supports a hypothesis that Ad.*mda-7* or GST-MDA-7 enhances the ratio of pro-apoptotic to anti-apoptotic proteins in cancer cells, thereby facilitating induction of apoptosis.

MDA-7/IL-24 and lysosomal dysregulation

Increased mitochondrial dysfunction caused by MDA-7/IL-24 has been linked to cytokine-induced ER stress; PERK signaling both suppresses the expression of MCL-1 and BCL-XL but also, via PERK-dependent increases in ROS/ceramide levels that cause activation of the JNK pathway which in turn promotes BAX and BAK activation; and this then promotes mitochondrial dysfunction [23,34,40] (Figure 1). In some cell types, notably only ovarian and renal carcinoma cells at present, MDA-7/IL-24 has been shown to cause activation of the extrinsic apoptosis pathway, in particular the death receptor CD95 [25,26]. In ovarian cancer cells, CD95 activation was ligand-independent and required MDA-7/IL-24–induced ceramide generation [25]. Downstream of the CD95 receptor, cleavage of BID again played a central role in mediating cytokine toxicity, though in ovarian and renal carcinoma cells BID cleavage is caspase 8-dependent, rather than cathepsin-dependent as was noted in glioblastoma cells.

MDA-7/IL-24 as a therapeutic tool

As noted previously, MDA-7/IL-24 is a secreted protein, and secreted MDA-7/IL-24 been shown in several studies to have a “toxic bystander” effect on distant tumor cells *in vitro*. From the standpoint of MDA-7/IL-24 as a gene therapeutic tool, based on simple mass action effects, it is not possible to infect every tumor cell within a tumor using an adenovirus even with intra-tumoral injection, and this has been one possible reason why so many gene therapy approaches have failed in the clinic. In addition, systemic IV administration of any

recombinant adenovirus will not result in productive infection of any disseminated tumor due to rapid first pass sequestration of virus by the liver coupled to neutralization by any pre-existing anti-adenovirus antibodies. Based on its selective and potent anti-cancer activity *in vitro* and in animal models, a Phase I clinical trial was performed in advanced carcinomas and melanomas using a replication incompetent serotype 5 adenovirus to express MDA-7/IL-24; Ad.*mda-7* (INGN-241) [41–45] (Table 1). These studies employed repeated intra-tumoral injection of Ad.*mda-7* in patients with advanced disease and indicated that repeated administration of Ad.*mda-7* was safe and this gene could induce apoptosis in a large percentage of tumor volume with a measurable clinical response rate of ~44%.

It was evident in this trial that infection of a small proportion of tumor cells with Ad.*mda-7* resulted in detectable MDA-7/IL-24 protein levels and increased tumor cell apoptosis many centimeters from the site of any virally infected tumor cell in the tumor, indicating, as was observed in animals, and now in patients, that secreted MDA-7/IL-24 was having a “*toxic bystander*” effect on uninfected tumor cells [46–49]. In addition, we have noted in both prostate, renal and glioblastoma xenograft tumors that Ad.*mda-7* infection of an established tumor growing on one flank of an animal results in growth arrest and apoptosis in an uninfected tumor growing on the opposite flank of the animal [49–51; unpublished results]. Clearly, however, at sites more distant to viral administration where MDA-7/IL-24 concentrations may be only growth inhibitory and not cytotoxic, the combination of MDA-7/IL-24 therapy with established therapeutic agents to enhance the toxicity of MDA-7/IL-24 would be of considerable utility. The toxic effects of MDA-7/IL-24 therapy could be combined with other treatment modalities to achieve an improved *profound* clinical response.

MDA-7/IL-24 radiosensitizes tumor cells

MDA-7/IL-24, delivered as either a virus, plasmid, GST-MDA-7/IL-24 or His6-MDA-7/IL-24, causes the generation of ROS in tumor cells, but not in non-transformed cells, and quenching of ROS production suppresses MDA-7/IL-24 toxicity. Several long-established therapeutic modalities also generate ROS in tumor cells as part of their toxic biology [52]. For example, ionizing radiation causes ionizing events in water, generating hydroxyl radicals that can impact on the function of mitochondria in cells, which in turn amplify the initial free radical signaling, generating large amounts of reactive oxygen and nitrogen species [53]. In addition, radiation can cause DNA damage, activate poly ADP ribosyl polymerase (PARP) leading to an altered cellular redox status due to NADPH depletion, which can also be sensed by mitochondria [54]. Radiation exposure also increases ceramide levels in tumor cells [55].

Radiotherapy is used as a primary modality for the treatment of many malignancies including those of the breast, brain, prostate, and lung. Based on the tumoricidal effects of both radiation and MDA-7/IL-24, it has been a logical step for investigators to determine whether MDA-7/IL-24 had radiosensitizing potential. Several laboratories have demonstrated that Ad.*mda-7*, GST-MDA-7/IL-24 and MDA-7/IL-24 can radiosensitize a wide variety of tumor cell lines *in vitro* and *in vivo* [56–60]. In studies using human glioma and prostate carcinoma cells, the ability of both ionizing radiation and MDA-7/IL-24 to generate reactive oxygen species (ROS) was directly linked to the radiosensitizing properties of MDA-7/IL-24. MDA-7/IL-24 activates ceramide synthase 6 as part of its toxic effects and ceramide synthase 6 was also linked to MDA-7/IL-24 toxicity; others have shown that radiotherapy utilizes ceramide synthase 6 to kill tumor cells [61]. Other therapeutic agents have also been shown to act, in part, by generating ROS, including arsenic trioxide, 4-hydroxyphenyl-retinamide (4-HPR), vitamin E and perillyl alcohol [62–68]. In general agreement with ROS enhancing the lethal actions of MDA-7/IL-24, combined treatment of

renal, brain, lung, breast, pancreatic and prostate carcinoma cells with MDA-7/IL-24 and in combination with radiotherapy or arsenic trioxide or 4-HPR resulted in a highly synergistic potentiation of tumor cell killing that was not manifested in non-transformed epithelial cell counterparts. Collectively, these findings argue that established and novel therapeutic modalities, which generate ROS can promote MDA-7/IL-24 lethality in cancer cells.

MDA-7/IL-24 regulates signaling pathways that control the apoptotic threshold and facilitate radiosensitization

The regulation of signal transduction pathway functions by Ad.*mda-7* and GST-MDA-7/IL-24 protein, particularly when combined with therapeutic modalities that generate ROS and ceramide, is as apparently complicated as the number of mechanisms by which MDA-7/IL-24 has been reported to induce cell death. As noted in a prior section, activation of STAT transcription factors does not appear to significantly modulate MDA-7/IL-24 lethality, despite MDA-7/IL-24 activating STAT transcription factors through IL-20 receptor complexes [14,15]. Data in several tumor cell types has argued that either Ad.*mda-7* or (GST-)MDA-7/IL-24 proteins promote activation of the p38 mitogen activated protein kinase (MAPK) pathway, which via GADD34 (CHOP) promotes growth arrest and cell death [69,70]. In part, this may be explained by data suggesting MDA-7/IL-24 causes PKR/PERK activation in some tumor cell types, which is a known up-stream activator of both p38 MAPK and GADD34. However, in some tumor cell types MDA-7/IL-24-induced p38 MAPK signaling clearly also plays a “switch-hitter” role with respect to growth and survival wherein low concentrations of MDA-7/IL-24 induce a level of p38 MAPK signaling that facilitates growth arrest and cell survival with higher MDA-7/IL-24 concentrations causing an intense sustained pathway activation that leads to tumor cell death [71]. Several studies have linked the c-Jun NH2-terminal kinase (JNK) pathway as a mediator of MDA-7/IL-24 toxicity; as MDA-7/IL-24 increases ROS and ceramide levels and as these messengers have been widely shown by many groups to strongly activate JNK pathway signaling, this finding is perhaps not too surprising [e.g. 72]. Other studies have demonstrated that MDA-7/IL-24 inhibits PI3K/AKT and ERK1/2 pathway function, which in the case of ERK1/2 signaling is mediated by MDA-7/IL-24-induced activation of PERK; this reduction in ERK1/2 activity further promotes the MDA-7/IL-24-induced reduction in MCL-1 levels and facilitates JNK pathway activation [71].

As a single agent ionizing radiation-induced cell killing in a variety of cancer cells has been linked to the activation of the JNK pathway [73,74]. When combined with ionizing radiation, MDA-7/IL-24 has been suggested to promote radiation toxicity by modulating JNK1/2/3 pathway signaling [59,75]. For example, lung cancer cells were radiosensitized by Ad.*mda-7* via JNK1/2 signaling, without radiation further enhancing MDA-7/IL-24-induced JNK1/2 activation [18,60]. Use of established rodent and human glioma cell lines, as well as primary human glioma cell isolates, demonstrated that Ad.*mda-7* caused radiosensitization *in vitro* and *in vivo*, and that *in vitro* sensitization was dependent on JNK1/2/3 activation and *in vivo* sensitization correlated with increased JNK1/2/3 phosphorylation [59,75,76]. Many groups have argued that prolonged intense JNK1/2/3 pathway signaling is involved in cell death processes.

Conclusions

MDA-7/IL-24 is a multi-faceted killer of cancer cells that has shown significant clinical benefit in patients as a single agent. Future clinical studies will be required to determine whether MDA-7/IL-24 represents a viable therapeutic in glioblastoma, and other cancers, and whether MDA-7/IL-24 can be rationally combined with other established cancer treatments to improve tumor control. Based on the remarkable efficacy shown by *mda-7/*

IL-24 using direct tumor injection in patients with advanced cancers, we are very optimistic that this molecule will display profound activity in patients with diverse cancers, especially when combined with therapeutic agents that promote ER stress responses. We are actively pursuing these combinatorial studies as well as investigating improved and unique ways of effectively delivering *mda-7/IL-24 in vivo*. One mechanism to increase the total amount of MDA-7/IL-24 being delivered to the site of the tumor is by use of a conditionally replicative adenovirus (CRAd) also termed cancer terminator viruses (CTV). A virus that only replicates in tumor cells will result in viral replication –dependent tumor cell killing as well as the synthesis and release of MDA-7/IL-24 that will kill and suppress the growth of uninfected tumor cells. Due to a lack of expression of the coxsackie and adenovirus receptor (CAR) many tumor cells cannot be infected by type 5 adenovirus and the development of viruses with chimeric knob proteins to deliver gene therapeutics is also being explored: a type 5/type 3 recombinant adenovirus to deliver MDA-7/IL-24 was recently shown by us to be a more effective therapeutic for GBM tumors *in vivo* than a “standard” type 5 virus [23]. Finally, it is possible that lethal though highly immunogenic forms of MDA-7/IL-24 e.g. GST-MDA-7, could be delivered to tumors via their encapsulation in microbubbles, that plus ultrasound, will target delivery of this cytokine to cancers [77]. Thus the possible approaches to deliver MDA-7/IL-24 are diverse in nature and all of the noted approaches will, we hope, be translated into the clinic for evaluation over the up-coming 5 years.

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Figure 1. Molecular pathways by which MDA-7/IL-24 regulates cell viability and cell growth
MDA/IL-24 has two major targets in cells: the IL-20/IL-22 receptor complexes and the HSP70 family chaperone GRP78/BiP. MDA-7/IL-24 binding to its cognate receptors activates STAT family transcription factors and activation of these factors can promote differentiation and proliferation in a cell type dependent manner. STAT transcription factors play no role in MDA-7/IL-24 toxicity. MDA-7/IL-24 binds to GRP78/BiP; it is possible that entry of bacterial synthesized GST-MDA-7 into tumor cells is mediated by binding to cell surface GRP78/BiP. The majority of GRP78/BiP is present in the endoplasmic reticulum and is bound to PKR-like endoplasmic reticulum kinase (PERK); the chaperone inhibits PERK kinase activity. MDA-7/IL-24 disrupts the association of GRP78/BiP with PERK permitting PERK to phosphorylate eIF2α; phospho-eIF2α suppresses the translation of the majority of cellular proteins resulting in the rapid loss of protective proteins that have short half lives such as MCL-1 and BCL-XL, and via ATF4 promotes the transcription of a specific subset of genes e.g. GADD34 that promote apoptosis. PERK signaling promotes increased LASS6 (ceramide synthase 6) levels that promote increased Ca²⁺ mobilization leading to elevated ROS levels. Increased ceramide/Ca²⁺/ROS activate JNK and p38 signaling that promotes activation of the toxic BH3 domain proteins BAX and BAK.

Table 1
Responses of patients to INGN-241 therapy (cohort 8)

Patients in cohort 8 were treated with 2×10^2 virus particles repeated $2 \times$ per week for 3 weeks. (a) Prior treatments: S, surgery; F, 5-fluorouracil; G, gemcitabine; IF, interferon γ ; IT, immuno-therapy; CDDP, cisplatin; RT, radiotherapy; T, Taxane. (b) SD, stable disease; CR, complete response; PR, partial response; PD, progressive disease. (c) Time in days from first injection of INGN-241 until death. (d) patients 81 and 86 did not complete one full course of treatment (6 injections). (e) Patient received 12 injections on compassionate use protocol.

Patient #	Gender/age	Diagnosis	Previous Treatments (a)	No. of injections	Response (b)	Time to Death (c)
81	M/71	Adeno-carcinoma	P, T, G	2 (d)	SD	nd
83	F/64	Melanoma	S, RT, IT, IF	24 (e)	CR, PR, SD	> 600
84	F/62	Melanoma	S, RT, IT, IF	12	PR	309
85	M/64	Penile Carcinoma	S, RT, CDDP, T	6	PD	75
86	F/66	NSCLC	RT, P, T	3 (d)	SD	180
87	M/62	SCCHN	S, RT, CDDP, T, F, P	6	PD	185
88	M/91	Lip carcinoma	S, RT	6	SD	181