# Review Article

---

## Conditions of tumor-associated antigens as a proper target for therapeutic antibodies against solid cancers

Yoshikazu Kurosawa

_____

Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan

## Abstract:

Since the success of rituximab and trastuzumab for treatment of non-Hodgkin's lymphoma and breast cancer, respectively, a huge therapeutic potential of monoclonal antibodies (mAbs) was realized and development of therapeutic mAbs has been widely tried against various cancers. However, the successful examples are still limited and therapeutic mAbs are not yet available for the majority of human cancers. We established a procedure for comprehensive identification of tumor-associated antigens (TAAs) through the extensive isolation of human mAbs that may become therapeutic. Thirty-twoTAAs have been identified and 555 mAbs that bound to one of the TAAs have been isolated to date. Now we are trying to select TAAs as proper targets for therapy and candidate mAbs as drugs from among them. The immunohistochemical analysis using many fresh lung cancer specimens suggested probabilities of proper targets, and moreover, presence of cancer-specific epitopes that could be distinguished from normal epitopes on the same molecules by mAbs. For Abs to efficiently kill the cancer cells they should have the ability to induce immunological cytotoxicity such as ADCC and/or CDC. They should also be able to inhibit the function mediated by the target Ags. For clinical point of view, the continuous presence of the target molecule on the cell surface until cell death might be essential for successful treatment. Therefore, it will be required for targets TAAs to play essential roles in tumorigenesis. Otherwise the cancer cells that do not express them could selectively survive during treatment and finally become dominant. It was also suggested that even the same molecules could play different roles in tumorigenesis quite often in different patients. Therefore when we develop therapeutic Abs, we should obtain information about the conditions of patients including genetic background to whom the treatment will be effective. I will discuss how we can accomplish this purpose.

### Introduction:

Since the success of rituximab and trastuzumab for treatment of non-Hodgkin's lymphoma and breast cancer, respectively, people realized a huge therapeutic potential of monoclonal antibodies (mAbs) and rushed into development of therapeutic Abs against cancers [1,2]. In the commentary "Magic bullets hit the target" published in Nature, June issue of 2002, Trisha Gura described the atmosphere of enthusiasm at that

time and pointed out the problem as follows [3]. "The biggest issue to be solved is cost. Although antibodies require much less investment in initial research and development than conventional small-molecule drugs, they are hugely expensive to manufacture." This comment presumed that many groups would succeed in development of therapeutic mAbs against cancers in a near future. However, in the paper "Colorectal cancer treatment: what's next?" published in 2008, six years later, Leonard Saltz described "although, initially, these new drugs appeared to offer enormous promise to radically change the landscape for patients with metastatic colorectal cancer, the passage of time has begun to show us that the advances that have been made, while real, are more modest than we had expected or hoped" and he argued "more sophisticated understanding of signal transduction pathways, and of immune surveillance and immunologically mediated cytotoxicity, will help to reveal potential therapeutic options for colorectal cancer" [4]. Thus, people started to think the necessity of a breakthrough for further revolutionary success in this field. The simple strategy composed of the following 4 steps: finding of tumor-associated antigens (TAAs), making mAbs against them, examining their antitumor activities *in vitro* and *in vivo* and starting clinical tests, may not result in the success in most cases.

The success of rituximab, anti CD20 mAb, and bevacizumab, anti VEGF mAb, indicated that the targets for therapeutic Abs are not necessarily TAAs that are located on the cell surface and preferentially expressed in malignant cells [5]. In addition to the use of unmodified IgG, the success of Zevalin, anti CD20 radiolabelled mAb, gave an alternative

way of the use of the Ab such as a delivery molecule to malignant cells [6]. In this review, however, I only focused on the TAAs that are preferentially overexpressed on the surface of malignant cells and could be effective targets by IgG type of therapeutic mAbs. According to these criteria only EGFR and HER2 are the antigens (Ags) against which therapeutic mAbs have already been approved by FDA to date and now are clinically available. While candidates for target Ags should be abundantly present on the surface of cancer cells and accessible by Abs, they should not be highly expressed on normal cells especially those that constitute vital organs. Thus, anticancer mAbs can distinguish malignant cells from normal cells. It is also required that they are homogeneously and consistently present on the surface of cancer cells. In this review I will summarize the strategies for identification of TAAs, the kinds of TAAs identified to date, classification of the TAAs, conditions of proper target TAAs and selection of effective mAbs as therapeutic drugs. Since we have already identified 29 TAAs and isolated 441 mAbs that bound to one of the TAAs to date [7], we mainly focused on these TAAs and mAbs in this review. Excellent reviews that treated the topics in more general way were given by other researchers [8-10].

## Strategies for identification of TAAs

Completion of human genome project allowed us to comprehensively examine all the proteins encoded on human genome. In order to identify TAAs, profiling of mRNAs present in tumor *vs* normal cells has been extensively performed using cDNA or oligonucleotide microarrays [11,12]. Although many candidate genes of TAAs

have been identified, this strategy implied the potential problems due to discordance between mRNA levels and proteins levels [13]. If tumor-associated splice variants were discovered [14], furthermore, if their roles in tumorigenesis became evident as known in the case of CD44v6 and EGFRvIII [15,16], they could be good TAAs recognized by therapeutic Abs.

Proteomic analysis could directly show which spots corresponded to TAAs on 2-dimensional (2D) gel. Development of mass spectrometry (MS) allowed us to identify proteins corresponding to respective spots even low in abundance. Since majority of the visible spots detected by the ordinary 2D gel technology were cytoplasmic proteins, however, further devices were required for enrichment of membrane proteins. Although membrane proteins could be selectively prepared by biotinylation of cell surface proteins in viable cells followed by affinity enrichment using streptavidin beads [17], this technology has not been successfully applied to identification of TAAs because of difficulty in quantitative preparation of all the proteins present on cell surface. A similar method was also reported. After biotinylation of membrane proteins they were purified by avidin affinity chromatography. The isolated proteins were applied to SDS-PAGE and visualized by silver staining. Then, bands were excised and identified by MS/MS analysis. Difference in abundance between cancer cells and normal cells was examined by HRP-avidin staining. Using this technology, Hastie et al. [18] succeeded in identification of several TAAs. In order to compare 2D gel images from different samples, DIGE method where samples were differently labeled prior to electrophoresis with spectrally resolvable

fluorescence dyes have been developed [19]. After isolation of membrane proteins from two different cells DIGE was applied for detection of differences in protein expression [20].

While there had been suspicion whether authentic cancer-specific Ags were present and immunogenic, therefore, recognized by the autologous host, development of the method termed SEREX (serological analysis of recombinant cDNA expression libraries) led to identification of many TAAs [21,22]. Based on the success of SEREX, sera from patients with cancer are used as the primary Ab to detect immunoreactive spots in 2D gel of malignant cell-derived proteins, resulting in identification of various TAAs including cancer/testis Ags [22].

In these strategies, identification of TAAs was performed first, and at the next step mAbs were prepared against them. When Ab technologies were utilized for identification of TAAs at an initial step, it became possible that the experiments were designed in the opposite direction [23]. MAbs against TAAs were isolated first and the target Ags would be identified at the next step. The details are described in the next section.

**Ab-directed identification of TAAs**

Given TAAs expressed on human cancer-derived cell lines are immunogenic in mice, mAbs against the TAAs could be generated by using conventional hybridoma technology. Since majority of the therapeutic Abs approved to date were derived from mouse mAbs, we may expect that mAbs isolated by this method could be utilized as a source of therapeutic Ab. According to this strategy, however, living cells expressing many molecules including TAAs were used as

Ag for immunization [23]. Since it is not easy to efficiently isolate many mAbs with different characteristics against a single TAA, this method would be useful in identification of membrane proteins whose amounts are abundant and that are accessible by Abs. Candidates for therapeutic mAbs would be isolated afterwards since various capabilities should be required for mAbs to efficiently kill cancer cells..

Using the phage-display Ab library we established a procedure for comprehensive identification of TAAs through the extensive isolation of human mAbs that may become therapeutic [7]. Our strategy was composed of four steps. First we isolated a large number of mAbs that bind to the surface of cancer cells using a huge phage Ab library and many kinds of cancer-derived cell lines. Then using fresh tumor tissues we selected clones that gave significant staining of malignant cells but were negative or very weakly positive on the normal cells in the histological sections. At the third step the Ags recognized by the respective clones were isolated by immunoprecipitation (IP) and identified by MS analysis. Finally mAbs were converted to complete $IgG_1$ and the antitumor activity was examined. Although several papers reporting identification of TAAs by using similar methods to ours were published [24-26], the number of TAAs identified as well as that of different kinds of mAbs bound each TAA were much less than those by our group. The quality of Ab library, the screening method, the definition of TAA for primary selection of clones, and the method of identification of Ags recognized by respective mAbs should have given various effects on the results. While we have already reported the method, termed ICOS, for comprehensive isolation of mAbs

specifically bound to membrane proteins [27], and the methods, termed GFC and SITE, for systematic identification of Ags recognized by many mAbs [28], I pointed out two specific issues here.

(1) The characteristics of clones isolated by screening were very much diverged among the libraries and appeared to be largely influenced by the source of B cells utilized in construction of Ab libraries. We used two different libraries in this project: AIMS, composed of $10^{11}$ independent clones and the B cells were derived from 25 umbilical cord bloods, 20 tonsils, 2 peripheral bloods and 1 bone marrow; AOCK, whose size was the same as that of AIMS but the B cell source was spleens from 4 patients with hepatocarcinomas. AIMS library gave us various mAbs with different characteristics specific against probably any kind of TAA but the repertoire of Abs formed in AOCK appeared to be much less and biased. $V_H$ genes encoding majority of the clones isolated from AIMS were germline-type without any mutation or with few. Although this result indicated they were naive clones to Ags, the *Kd* values between mAbs isolated and the target TAAs were high enough, between 0.1 to 10 nM, in most cases.

(2) When we started this project, we presumed that many of the clones isolated might bind to Ags commonly present on both malignant and normal cells, therefore, number of anti TAA mAbs could not be many. Probably this notion could have been commonly shared among researchers, and in fact several groups selected clones based on the restricted criteria of effective targets for therapeutic Abs during the screening process before identification of Ags [24-26]. Thus, they picked up only limited number of clones at the final stage for identification of TAAs. For example, many

groups that looked for TAAs used normal tissue-derived cell lines as negative controls. According to our experience, when normal tissue-derived cells were established as cell lines they had to gain the ability of immortalization. Since the genes involved in immortalization largely overlapped with those necessary for tumorigenesis, they judged possible TAAs as normally expressed genes. As long as the membrane proteins that are expressed on the cell surface of immortalized cell lines are not expressed on the normal tissues under the steady state conditions *in vivo*, they could be proper targets by therapeutic Abs. We selected the clones only based on the immunohistochemical (IHC) analysis of fresh cancer tissues. Then around one third of mAbs isolated were judged to give tumor-specific staining patterns [7].

.

**TAAs identified by various methods**

It is not so simple to define TAA commonly approved among researchers. Since chimeric proteins such as Bcr-Abl fusion protein generated by chromosome translocation resulting in chronic myelogenous leukemia (CML) are not present in any normal cells, they are genuinely cancer-specific. But this example appeared to be very rare in pathogenesis of cancers. If introduction of mutations into some protein caused oncogenicity, we could consider it a cancer-specific molecule. However, it will not be practically easy to prepare mAbs that can distinguish the mutated protein from normal one. From practical point of view to developing therapeutic Abs, the important points would be how we identify target TAAs on the surface of cancer cells that play essential roles in tumorigenesis and how we prepare mAbs that could inhibit their function. As a

target recognized by therapeutic Abs, the continuous presence on the cell surface until cell death might be essential for successful treatment. For example in the case of proteins classified into cancer/testis Ag, it does not seem to be evident whether they are directly involved in tumorigenesis or they are expressed in cancer cells by dysregulation of transcription without any specific role in tumorigenesis. If the latter is the case, it would be possible that the cancer cells that do not express them could selectively survive during treatment and finally become dominant. Since transcription is regulated mainly at epigenetic level, such cells resistant to the treatment with therapeutic Ab might start to appear at some frequency.

Judging from their predicted function, majority of the TAAs identified according to our Ab-directed strategy appeared to play some role in tumorigenesis. We have identified 32 membrane proteins as TAAs and listed in Table 1. While many of them could not be tumor-specific in the strict meaning because of expression in normal tissues, the characteristics of these proteins commonly observed among them were: 1. they were abundantly present on the cell surface of not only some cancer-derived established cell lines but also some fresh cancers; 2. The mAbs against them gave tumor-specific staining patterns in IHC analysis of some cancer tissues freshly resected, 3. Overexpression of these TAAs were observed not in specific cancers but in various kinds of carcinomas. We roughly estimated that the total number of TAAs that showed the characteristics indicated here and that could be potentially identified by our method might be 40 kinds in total.

## TAAs (32kinds) identified and human mAbs (555 kinds) isolated to date

| Growth factor receptor | | Adhesion molecule (IgSF) | | Adenosine metabolism | |
|---|---|---|---|---|---|
| EGFR | 12 | CADM1/IGSF4 | 29 | CD73 | 1 |
| HER2 | 22 | ALCAM/CD166 | 13 | Complement inhibitor | |
| HGFR | 84 | ICAM-1/CD54 | 25 | MCP | 101 |
| PTK 7 /CCK-4 | 1 | Lu/BCAM | 49 | Protease inducer | |
| EphA2 | 26 | CEACAM6 | 1 | EMMPRIN | 5 |
| IGF1R | 3 | CEA | 2 | Prostate-specific antigen | |
| PDGFR | 3 | JAM-A | 2 | PSMA | 1 |
| Tyrosine phosphatase | | MCAM/MUC18/CD146 | 22 | Iron metabolism | |
| PTP-LAR | 46 | Adhesion molecule (Non IgSF) | | TfR | 15 |
| Integrin family | | CD44 | 13 | Apoptosis inducer | |
| α3β1 | 16 | EpCAM | 3 | TRAILR2 | 7 |
| αVβ3 | 7 | Tetraspanin | | Complement receptor | |
| α6β4 | 5 | CD9 | 1 | C1qR | 2 |
| α2β1 | 1 | Tetraspanin partner | | | |
| Anoikis regulator | | PTGFRN(CD9P-1) | 3 | | |
| CDCP1 | 31 | | | | |

## Classification of TAAs

The 32 TAAs identified to date were classified into 15 groups in Table 1 according to the predicted function. The largest group among them was cell adhesion molecules belonging to immunoglobulin superfamily (IGSF). While it has been shown that they play roles in cell-cell adhesion, they are also committed to some other phenomena. Although CADM1 had been originally identified to be tumor suppressor [33], we showed it could also function as oncogene in lung adenocarcinoma [34]. While the roles of these adhesion molecules in the tumorigenesis have not yet been precisely revealed in most cases and they are expressed on various normal cells, several groups are trying to develop therapeutic

mAbs to these molecules based on various strategies [35-41]

According to the sequence data obtained by human genome project, the total number of receptor tyrosine kinase (RTKs) was estimated to be 58 [42]. Among them seven RTKs including EGFR and HER2 have been identified to be TAAs by our method. Since PTK7/CCK4 is a pseudokinase and the ligand has not been identified, it might be difficult to examine the role of this molecule in tumorigenesis [43]. Since it has been well established that phosphorylation mediated by protein tyrosine kinases (PTK) plays a crucial role in control of cell growth and that PTKs play an important role in almost all types of cancer, not only EGFR and HER2 but also IGF1R, HGFR and EphA2

could be good targets for therapeutic Abs [44,45].

Tyrosine phosphorylation should be controlled by a balance of PTKs and protein tyrosine phosphatases (PTP). Since an overexpression of PTKs has been shown to contribute to carcinogenesis, it seemed to be reasonable to argue that PTPs may function as tumor suppressor opposite to that mediated by PTKs [46]. However, PTP-LAR was identified to be TAA while 22 different receptor-type PTPs have been identified in human genome [47]. Since PTPs and PTKs interacted with each other not only functionally but also physically [48], there will be various ways to induce perturbation in the complexes by using Abs. Therefore, it might be a good candidate as the target for therapeutic Abs. So far there has been no report of developing therapeutic Abs to this protein.

Integrins are heterodimeric receptors consisting of $\alpha$ and $\beta$ subunits. In mammals, 18 $\alpha$ and 8 $\beta$ subunits assemble 24 different receptors [49]. Integrins interact with various extracellular matrix (ECM) components such as laminin, collagen and fibronectin and mediate cell-ECM adhesion. Such interactions give the strong effects on cellular behavior through determination of structural features of a tissue and transmission of signals from the surrounding microenvironment to epithelial cells. Of the 24 integrins four have been identified as TAAs. Since mAbs against $\alpha3\beta1$ have been most frequently isolated by our method and it is abundantly expressed at high frequency on the cancer cell surface, it seemed to be evident that it plays a crucial role in tumorigenesis. This argument is supported by the experiments with siRNA. Cell growth of various tumor cell lines could be inhibited by siRNA of $\alpha3$ and of $\beta1$ (unpublished results). However, since it is

also well expressed on the various normal cell surfaces, it will be difficult to develop therapeutic Abs without adverse effects on normal organs. It has been known that $\alpha v\beta3$ is highly expressed on mature osteoclasts, angiogenic endothelial cells and tumor cells, and development of therapeutic Abs against $\alpha v\beta3$ was already started [50,51]. Both $\alpha3\beta1$ and $\alpha6\beta4$ were major cellular receptors for laminin 5, their functions were different from each other. Our IHC analysis suggested that $\alpha6\beta4$ could be a proper target for therapeutic Abs [52]. Trial for the development of therapeutic mAbs to $\alpha6\beta4$ was reported [53].

Tetraspanins are cell-surface proteins that span the membrane four times and form a large family consisting of 32 members [54]. While some of them were identified as TAAs [55], our TAA collection included CD9. Since tetraspanins are present on various normal cell surfaces, they will not be proper targets by therapeutic mAbs. From a different point of view, however, their function is a molecular organizer of multi-protein complexes, located at the specific region termed tetraspanin-enriched microdomains (TEMs), and it is involved in cellular signaling [56]. Since integrins, CD44 and tetraspanins are deeply involved in the signal pathway through forming a network of large complexes with various signal molecules, it would be possible to find a tumor-specific complex that could be distinguished by mAbs [57,58]. Although CD44v6 has been well known to be TAA, the phase I trial with the immunoconjugate consisting of a toxin coupled to anti CD44v6 mAb recently failed because of serious skin toxicity [59].

Ecto-5'-nucleotidase (CD73) is a GPI-anchored cell-surface protein and catalyzes the extracellular conversion of 5'-AMP to adenosine. While RNA

interference of CD73 suggested that it may have multiple functions independent of the enzyme activity [60], we found that anti CD73 mAb efficiently prevented cell growth (unpublished results). Although this molecule is found on the surface of a variety of cell types, it might be a target by therapeutic Abs if the cancer-specific function could be revealed [61].

The transferrin receptor (TfR) has been one of the most hopeful targets by therapeutic mAbs [62,63]. The role of transferrin (Tf) is to transport iron through the blood and deliver it to cells through TfR. Therefore, TfR is expressed in normal tissues including liver, epidermis, intestinal epithelium and a part of blood cells in the bone marrow. But the expression level is low in general. On the other hand, on the cell surface of many tumors, extremely high expression was observed. Moreover, some mAbs showed strong activity of cell killing. In our case, for instance, the cells producing an anti TfR mAb could not grow under ordinary culture conditions because of cell killing by the Ab secreted from the cells themselves (unpublished results). Clinical trials using several candidates for therapeutic mAbs are ongoing.

Although involvement of CDCP1 in tumorignesis was recently discovered, it could be possible to develop therapeutic Abs to it in a near future. Since a precise review on CDCP1 was just published [64], I pointed out only the possibility of therapeutic Abs here. While this molecule plays a key role in the regulation of anoikis, phosphorylation of conserved tyrosine residues at the cytoplasmic domain of CDCP1 confers resistance of cancer cells to anoikis [65]. Moreover, since CDCP1 interacts with many key proteins including integrins, tetraspanins and Src family kinases, analyses of antitumor activity mediated by anti CDCP1 mAbs could be

precisely performed [64]. In fact development of therapeutic Abs to CDCP1 has already been started [66]

Membrane cofactor protein (MCP)/CD46, decay-accelerating protein (DAF)/CD55 and CD59 are well known to be membrane-associated complement-inhibitory proteins that protect normal tissues from damage by autologous complement. All of these three molecules were frequently overexpressed on malignant cells and overexpression of CD46 was especially distinct [67]. While the fact that the complement inhibitors are overexpressed on tumor cells appears to imply presence of autologous Abs against TAAs in patients with cancers, it would be possible that MCP might play an additional role in tumorigenesis. Expression of siRNA to MCP gave an inhibitory effect on the growth of tumor cells under complement-free conditions *in vitro* (unpublished results).

Since extracellular matrix metalloproteinase inducer (EMMPRIN)/CD147 containing two Ig superfamily domains induced production of MMP-1 and MMP-2 by surrounding fibroblasts, it has been argued that overexpression of this molecule gave tumor cells the ability to grow under anchorage-independent conditions [68]. However, it is now clear that EMMPRIN is a multifunctional protein and interacts with a wide range of binding partners [69]. SiRNA experiment indicated that EMMPRIN is indispensable for the growth of tumor cells [70]. Since the IHC analysis gave tumor-specific staining patterns (unpublished results), EMMPRIN should be a good target for therapeutic mAbs.

## Lessons from success of trastuzumab and cetuximab

It has been shown that the ability of Abs inducing immune responses initiated

by Ag/Ab complexes such as AADC and CDC is very important for therapeutic Abs to efficiently kill the cancer cells [71,72]. In the case of TAAs identified by our strategy, however, around a half of the mAbs isolated showed AADC against the tumor cells that expressed the abundant target TAAs [7]. It may be due to the following characteristics of the TAAs identified by our method: they are abundantly present on the tumor cell surface and easily accessible by Abs. While anti EpCAM mAb showed an extremely strong AADC activity *in vitro* [7], in the xenograft model using nude mice the activity was largely dependent on the conditions of target tumor cells. When the Ab was injected into mice together with cancer cells, it showed a strong antitumor activity. But if it was injected after the cancer cells formed a certain volume of solid tumor, it showed only a weak antitumor activity (unpublished results). Thus, the ability to induce AADC is one of the conditions necessary for therapeutic Abs but not enough.

Crystallographic studies convincingly revealed how EGFR is regulated by the ligands [73]. Furthermore, structural analysis of the complex between cetuximab and EGFR clearly indicated the molecular mechanism of inhibition of the ligand binding by cetuximab [74]. While it primarily inhibits the ligand binding, inhibits receptor dimerization and thus blocks autophosphorylation of EGFR and activation, it also induces receptor internalization as occurs with binding of the natural ligand. Activation of EGFR leads to stimulation of various signaling pathways including pI3K-AKT, MAPK, SRC and STAT by recruiting proteins to specific phosphorylated tyrosine residues in the C terminal domain [75]. Since EGFR overexpressed on the tumor cells plays essential roles in tumorigenesis such as

cell motility, invasiveness and metastasis through the stimulation of these signaling pathways, cetuximab eventually inhibits all of these phenomena mediated by EGFR. However, there are many ways for cancer cells to show resistance to EGFR-directed therapeutics [75]. We isolated 12 kinds of anti EGFR mAbs as indicated in Table 1. Using all of them plus cetuximab and panituzumab we systematically examined the antitumor activity such as inhibition of cell growth *in vitro* and *in vivo* against various cell lines. The results indicated the followings: all of them showed antitumor activity with various intensities; the combinations of target cells and mAbs which showed either the strong activity or less activity were very variable (unpublished results). It suggested that while EGFR mediated various kinds of activity, there could be differences in the activities which respective tumor cells utilized in tumorigenesis. There could also be differences in the activities which respective mAbs could inhibit. It has been shown that patients with a colorectal tumor bearing mutated *K-ras* did not benefit from cetuximab, where patients with a tumor bearing wild-type *K-ras* did benefit from cetuximab [76]. It could be possible that some other mAb than cetuximab might show antitumor activity even against tumors with mutated *K-ras*.

HER2 gave a good example of successful therapeutic mAbs. Trastuzumab binds to the juxtamebrane region of HER2 [77] and shows a strong antitumor activity through blocking the various functions mediated by HER2 in addition to ADCC [78]. Pertuzumab also binds to HER2 near the center of domain II that contains a dimerization arm and inhibits dimerization with HER1 or HER3 [79]. Thus, it has been expected that trastuzumab and pertuzumab will be effective in the treatment of different types of cancers

[80]. Since there seem to be various pathways through which resistance to the established therapy is obtained [78], different options for treatment of cancers should be developed even against a single target molecule.

## Search for proper targets and selection of candidates clones

In order to search for target Ags and to select the candidates of therapeutic Abs to solid cancers we started the following systematic studies. 1. IHC analysis using large number of fresh cancer tissues; 2. Examination of antitumor activities, *in vitro* (ADCC, inhibitory effects on cell growth) and *in vivo* (xenograft using nude mice); 3. SiRNA of TAAs. Although the experiments have not been finished yet, we could argue the followings. The IHC analyses were performed using 36 fresh lung cancer specimens resected from patients. Thee portions in lung, tumor cells, bronchial epithelial cells and normal pulmonary alveolus cells including interalveolar septum were stained and compared each other. The results were very informative since bronchial epithelial cells appeared to represent the case where cells are frequently turned over under healthy steady state conditions. In fact EGFR was expressed not only on the cancer cell surface but also on the surface of bronchial epithelial cells although at low level. Based on the results of IHC we concluded that HGFR, PTP-LAR, EMMPRIN, TfR, CDCP1, integrin $\alpha v \beta 3$ and integrin $\alpha 6 \beta 4$ could be proper targets in addition to EGFR and HER2 [52]. One more intriguing observation was obtained from IHC analysis. While we isolated multiple clones against respective TAAs, they appeared to bind different epitopes on the single TAA molecule from each other. As we already reported such examples using anti CADM1 mAbs [34], it

suggested presence of a "cancer-specific epitope" that could be distinguished from normal epitopes.

Although cancers are very heterogeneous diseases, there should be rules that govern the transformation of normal cells into malignant cancers. Hanahan and Weinberg [81] proposed six essential capabilities required for cancers. When normal cells newly obtained one of the abilities during the process of tumorigenesis, for example self-sufficiency in growth signals, there should be many potential genes responsible for such an activity which would be mutated or whose expression might be up-regulated or down-regulated. Furthermore, even one target gene could be involved in tumorigenesis in various ways. For example, if we were able to precisely analyze various cancer cells in which EGFR was overexpressed, we would know the roles of EGFR in tumorigenesis could be different among them. Therefore, when we develop therapeutic Abs, we should also obtain information about the conditions of patients to whom the treatment with the Abs will be effective. This means that we should develop various options since the patients who can benefit from the treatment with a therapeutic Ab will be restricted.

## Perspective

In xenograft model using nude mice we have already had many examples where mAbs showed strong antitumor activities. As I described examples of anti EGFR mAbs, the combinations of cancer cells and mAbs that gave strong antitumor activity were very heterogeneous. Differences in response to the treatment may be mainly derived from the differences in genetic background including the epigenetic level. Moreover, we observed many examples showing

presence of a "cancer-specific epitope" recognized by mAbs. It is possible that such epitopes reflected presence of "cancer-specific complexes". In order to overcome the difficulty in clearing the final clinical tests in which many patients randomly divided to two groups, candidate Ab *vs* placebo, have to be treated, two things should be done before starting large scale clinical trials. The factors that determine the effectiveness of therapeutic Abs should be precisely examined. Based on the informed consent between patients and clinical doctors, the patients that may be curable would be searched and tested. This information allowed us to establish the rationale for selection of patients. This can be performed only as a clinical research termed "translational research" by tight collaboration between basic scientists and clinical doctors.

*Acknowledgments*

**References**:

1) Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 1994;83:435-45.

2) Carter P, Presta L, Gorman CM, Ridgway JBB, Henner D, Wong WLT, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of anti-p185$^{HER2}$ antibody for human cancer therapy. Proc Natl Acad Sci USA 1992;89:4285-9.

3) Gura T. Magic bullets hit the target. Nature 2002;417:584-6.

4) Saltz L. Colorectal cancer treatment: what's next? (or: Is there life after EGFR and VEGF?) Gastrointest Cancer Res 2008;2:S20-2.

5) Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 2004;3:391-400.

6) Bennett JM, Kaminski MS, Leonard JP, Vose JM, Zelenetz AD, Knox SJ, Horning S, Press OW, Radford JA, Kroll SM, Capizzi RL. Assessment of treatment-related myelodysplastic syndromes and acute myeloid leukemia in patients with non-Hodgkin lymphoma treated with tositumomab and iodine I$^{131}$ tositumomab. Blood 2005;105:4576-82.

7) Kurosawa G, Akahori Y, Morita M, Sumitomo M, Sato N, Muramatsu C, Eguchi K, Matsuda K, Takasaki A, Tanaka M, Iba Y, Hamada-Tsutsumi S, Ukai Y, Shiraishi M, Suzuki K, Kurosawa M, Fujiyama S, Takahashi N, Kato R, Mizoguchi Y, Shamoto M, Tsuda H, Sugiura M, Hattori Y, Miyakawa S, Shiroki R, Hoshinaga K, Hayashi N, Sugioka A, Kurosawa Y. Comprehensive screening for antigens overexpressed on carcinomas via isolation of human mAbs that may be therapeutic. Proc Natl Acad Sci USA 2008;105:7287-92.

8) Carter P, Smith L, Ryan M. Identification and validation of cell surface antigens for antibody targeting in oncology. Endocr Relat Cancer 2004;11:659-87.

9) Reichert JM, Valge-Archer VE. Development trends for monoclonal antibody cancer therapeutics. Nat Rev Drug Discov 2007;6:349-56.

10) Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. CA Cancer J Clin 2009;59:111-37.

11) Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: Identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001;61:2129-37.

12) Hippo Y, Taniguchi H, Tsutsumi S, Machida N, Chong JM, Fukayama M, Kodama T, Aburatani H. Global gene expression analysis of gastric cancer by oligonucleotide microarrays. Cancer Res 2002;62:233-40.

13) Chen G, Gharib TG, Huang CC, Taylor JMG, Misek DE, Kardia SLR, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, Beer DG. Discordant protein and mRNA expression

in lung adenocarcinomas. Mol Cell Proteomics 2002;1:304-13.

14) Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science 2003;302:2141-4.

15) Rudy W, Hofmann M, Schwartz-Albiez R, Zoller M, Heider KH, Ponta H, Herrlich P. The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually suffices to confer metastatic behavior. Cancer Res 1993;53:1262-8.

16) Sugawa N, Ekstrand AJ, James CD, Collins VP. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. Proc Natl Acad Sci USA 1990;87:8602-6.

17) Zhao Y, Zhang W, Kho Y, Zhao Y. Proteomic analysis of integral plasma membrane proteins. Anal Chem 2004;76:1817-23.

18) Hastie C, Saxton M, Akpan A, Cramer R, Masters JR, Naaby-Hansen S. Combined affinity labelling and mass spectrometry analysis of differential cell surface protein expression in normal and prostate cancer cells. Oncogene 2005;24:5905-13.

19) Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 2003;3:36-44.

20) Dowling P, Meleady P, Dowd A, Henry M, Glynn S, Cynes M. Proteomic analysis of isolated membrane fractions from superinvasive cancer cells. Biochim Biophys Acta 2007;1774:93-101.

21) Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 1995;92:11810-3.

22) Lee SY, Jeoung D. The reverse proteomics for identification of tumor antigens. J Microbiol Biotechnol 2007;17:879-90.

23) Loo DT, Mather JP. Antibody-based identification of cell surface antigens: targets for cancer therapy. Curr Opin Phamacol 2008;8:627-31.

24) Geuijen CAW, Bijl N, Smit RCM, Cox F, Throsby M, Visser TJ, Jongeneelen MAC, Bakker ABH, Kruisbeek AM, Goudsmit J, de Kruif J. A proteomic approach to tumor target identification using phage display, affinity purification and mass spectrometry. Eur J Cancer 2005;41:178-87.

25) Goenaga AL, Zhou Y, Legay C, Bougherara H, Huang L, Liu B, Drummond DC, Kirpotin DB, Auclair C, Marks JD, Poul MA. Identification and characterization of tumor antigens by using antibody phage display and intrabody strategies. Mol Immunol 2007;44:3777-88.

26) Siva AC, Kirkland BE, Lin B, Maruyama T, McWhirter J, Yantiri-Wernimont F, Bowdish KS, Xin H. Selection of anti-cancer antibodies from combinatorial libraries by whole-cell panning and stringent subtraction with human blood cells. J Immunol Meth 2008;330:109-19.

27) Akahori Y, Kurosawa G, Sumitomo M, Morita M, Muramatsu C, Eguchi K, Tanaka M, Suzuki K, Sugiura M, Iba Y, Sugioka A, Kurosawa Y. Isolation of antigen/antibody complexes through organic solvent (ICOS) method. Biochem Biophys Res Commun 2009;378:832-5.

28) Kurosawa G, Sumitomo M, Akahori Y, Matsuda K, Muramatsu C, Takasaki A, Iba Y, Eguchi K, Tanaka M, Suzuki K, Morita M, Sato N, Sugiura M, Sugioka A, Hayashi N, Kurosawa Y. Methods for comprehensive identification of membrane proteins recognized by a large number of monoclonal antibodies. J Immunol Meth (submitted).

29) Pollak M. Insulin and insulin-like growth factor signaling in neoplasia. Nat Rev Cancer 2008;8:915-28.

30) Oei ALM, Sweep FCGJ, Thomas CMG, Boerman OC, Massuger LFAG. The use of monoclonal antibodies for the treatment of epithelial ovarian cancer. Int J Oncol 2008;32:1145-57.

31) Cid C, Regidor I, Poveda PD, Alcazar A. Expression of heat shock protein 90 at the cell surface in human neuroblastoma cells. Cell Stress Chaperones 2009;14:321-7.

32) Smith LM, Nesterova A, Ryan MC, Duniho S, Jonas M, Anderson M, Zabinski RF, Sutherland MK, Gerber HP, van Orden KL, Moore PA, Ruben SM, Carter PJ. CD133/prominin-1 is a potential therapeutic target for antibody-drug conjugates in hepatocellular and gastric cancers. Br J Cancer

2008;99:100-9.

33) Kuramachi M, Fukuhara H, Nobukuni T, Kanbe T, Maruyama T, Ghosh HP, Pletcher M, Isomura M, Onizuka M, Kitamura T, Sekiya T, Reeves RH, Murakami Y. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. Nature Genet 2001;27:427-30.

34) Kitamura Y, Kurosawa G, Tanaka M, Sumitomo M, Muramatsu C, Eguchi K, Akahori Y, Iba Y, Tsuda H, Sugiura M, Hattori Y, Kurosawa Y. Frequent overexpression of CADM1/IGSF4 in lung adenocarcinoma. Biochem Biophys Res Commun 2009;383:480-4.

35) Roth A, Drummond DC, Conrad F, Hayes ME, Kirpotin DB, Benz CC, Marks JD, Liu B. Anti-CD166 single chain antibody-mediated intracellular delivery of liposomal drugs to prostate cancer cells. Mol Cancer Ther 2007;6:2737-46.

36) Brooks KJ, Coleman EJ, Vitetta ES. The antitumor activity of an anti-CD54 antibody in SCID mice xenografted with human breast, prostate, non-small cell lung, and pancreatic tumor cell lines. Int J Cancer 2008;123:2438-45.

37) Kikkawa Y, Sudo R, Kon J, Mizuguchi T, Nomizu M, Hirata K, Mitaka T. Laminin 5 mediates ectopic adhesion of hepatocellular carcinoma through integrins and/or Lutheran/basal cell adhesion molecule. Exp Cell Res 2008;314:2579-90.

38) Riley CJ, Engelhardt KP, Saldanha JW, Qi W, Cooke LS, Zhu Y, Narayan ST, Shakalya K, Croce KD, Georgiev IG, Nagle RB, Garewal H, von Hoff DD, Maradevan D. Design and activity of a murine and humanized anti-CEACAM6 single-chain variable fragment in the treatment of pancreatic cancer. Cancer Res 2009;69:1933-40.

39) Strickland LA, Ross J, Williams S, Ross S, Romero M, Spencer S, Erickson R, Sutcliffe J, Verbeke C, Polakis P, van Bruggen N, Koeppen H. Preclinical evaluation of carcinoembryonic cell adhesion molecule (CEACAM) 6 as potential therapy target for pancreatic adenocarcinoma. J Pathol 2009;218:380-90.

40) Bidlingmaier S, He J, Wang Y, An F, Feng J, Barbone D, Gao D, Franc B, Broaddus VC, Liu B. Identification of MCAM/CD146 as the target antigen of a human monoclonal antibody that recognizes both epithelioid and sarcomatoid types of mesothelioma. Cancer Res 2009;69:1570-7.

41) McSherry EA, McGee SF, Jirstrom K, Doyle EM, Brennan DJ, Landberg G, Dervan PA, Hopkins AM, Gallagher WM. JAM-A expression

positively correlates with poor prognosis in breast cancer patients. Int J Cancer 2009;125:1343-51.

42) Muller-Tidow C, Schwable J, Steffen B, Tidow N, Brandt B, Becker K, Schulze-Bahr E, Halfter H, Vogt U, Metzer R, Schneider PM, Buchner T, Brandts C, Berdel WE, Serve H. High-throughput analysis of genome-wide receptor tyrosine kinase expression in human cancers identifies potential novel drug targets. Clin Cancer Res 2004;10:1241-9.

43) Boudeau J, Miranda-Saavedra D, Barton GJ, Alessi DR. Emerging roles of pseudokinases. Trend Cell Biol 2006;16:443-52.

44) Jin H, Yang R, Zheng Z, Romero M, Ross J, Bou-Reslan H, Carano RAD, Kasman I, Mai E, Young J, Zha J, Zhang Z, Ross S, Schwall R, Colbern G, Merchant M. MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. Cancer Res 2008;68:4360-8.

45) Jackson D, Gooya J, Mao S, Kinneer K, Xu L, Camara M, Fazenbaker C, Fleming R, Swamynathan S, Meyer D, Senter PD, Gao C, Wu H, Kinch M, Coats S, Kiener PA, Tice DA. A human antibody-drug conjugate targeting EphA2 inhibits tumor growth in vivo. Cancer Res 2008;68:9367-74.

46) Muller T, Choidas A, Reichmann E, Ullrich A. Phosphorylation and free pool of -catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J Biol Chem 1999;274:10173-83.

47) Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Meller NPH. Structural and evolutionary relationships among protein tyrosine phosphatase domains. Mol Cell Biol 2001;21:7117-36.

48) Vogel W, Lammers R, Huang J, Ullrich A. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. Science 1993;259:1611-4.

49) Humphries MJ, McEwan PA, Barton SJ, Buckley PA, Bella J, Mould AP. Integrin structure: heady advances in ligand binding, but activation still makes the knees wobble. Trend Biochem Sci 2003;28:313-20.

50) Delbaldo C, Raymond E, Vera K, Hammershaimb L, Kaucic K, Lozahic S, Marty M, Faivre S. Phase I and pharmacokinetic study of etaracizumab (Abegrin™), a humanized monoclonal antibody against v 3 integrin receptor, in patients with advanced solid tumors. Invest New Drugs 2008;26:35-43.

51) Ning S, Nemeth JA, Hanson RL, Forsythe K, Knox SJ. Anti-integrin monoclonal antibody CNTO 95 enhances the therapeutic efficacy of fractionated radiation therapy in vivo. Mol Cancer Ther 2008;8:1569-78.

52) Kurosawa G, Tanaka M, Morita M, Sumitomo M, Sato N, Muramatsu C, Eguchi K, Akahori Y, Miyakawa S, Uyama I, Shiroki R, Hoshinaga K, Mizoguchi Y, Tsuda H, Hattori Y, Sugioka A, Sugiura M, Kurosawa Y. HGFR, PTP-LAR, EMMPRIN, TfR, CDCP1, Integrin v 3 and Integrin 6 4 could be proper targets for therapeutic antibodies against lung cancer. Cancer Res (submitted).

53) Dydensborg AB, Teller IC, Grulx JF, Basora N, Pare F, Herring E, Gauthier R, Jean D, Beaulieu JF. Integrin 6B 4 inhibits colon cancer cell proliferation and c-Myc activity. BMC Cancer 2009;9:223 published online.

54) Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define novel type of membrane microdomain. Annu Rev Cell Dev Biol 2003;19:397-422.

55) Boucheix C, Duc GHT, Jasmin C, Rubinstein E. Tetraspanins and malignancy. Exp Rev Mol Med 2001 http://www-ermm.cbcu.cam.ac.uk/01002381h.htm

56) Claas C, Wahl J, Orlicky DJ, Karaduman H, Schnolzer M, Kempf T, Zoller M. The tetraspanin D6.1A and its molecular partners on rat carcinoma cells. Biochem J 2005;389:99-110.

57) Palyi-Krekk Z, Barok M, Kovacs T, Saya H, Nagano O, Szollosi J, Nagy P. EGFR and ErbB2 are functionally coupled to CD44 and regular shedding, internalization and motogenic effect of CD44. Cancer Lett 2008;263:231-42.

58) Kuhn S, Koch M, Nubel T, Ladwein M, Antolovic D, Klingbeil P, Hildebrand D, Moldenhauer G, Langbein L, Franke WW, Weitz J, Zoller M. A complex of EpCAM, Claudin-7, CD44 variant isoforms, and tetraspnins promotes colorectal cancer progression. Mol Cancer Res 2007;5:553-67.

59) Riechelmann H, Sauter A, Golze W, Hanft G, Schroen C, Hoermann K, Erhardt T, Gronau S. Phase I trial with the CD44v6-targeting immunoconjugate bivatuzumab mertansine in head and neck squamous cell carcinoma. Oral Oncol 2008;44:823-9.

60) Zhi X, Chen S, Zhou P, Shao Z, Wang L, Ou Z, Yin L. RNA interference of ecto-5'-nucleotidase (CD73) inhibits human breast cancer cell growth and invasion. Clin Exp Metastasis 2007;24:439-48.

61) Colgan SP, Eltzschig HK, Eckle T, Thompson LF. Physiological roles for ecto-5'-nucleotidase (CD73). Pur Signal 2006;2:351-60.

62) Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML. The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin Immunol 2006;121:144-58.

63) Daniels TR, Delgado T, Helguera G, Penichet ML. The transferrin receptor part II: Targeted delivery of therapeutic agents into cancer cells. Clin Immunol 2006;121:159-76.

64) Wortmann A, He Y, Deryugina EI, Quigley JP and Hooper JD. The cell surface glycoprotein CDCP1 in cancer- insights, opportunities, and challenges. IUBMB Life 2009;61:723-30.

65) Uekita T, Jia L, Narisawa-Saito M, Yokota J, Kiyono T, Sakai R. CUB domain-containing protein 1 is a novel regulator of anoikis resitance in lung adenocarcinoma. Mol Cell Biol 2007;27:7649-60.

66) Siva AC, Wild MA, Kirkland RE, Nolan MJ, Lin B, Maruyama T, Yantiri-Wernimont F, Frederickson S, Bowdish KS, Xin H. Targeting CUB domain-containing protein 1 with a monoclonal antibody inhibits metastasis in a prostate cancer model. Cancer Res 2008;68:3759-66.

67) Rushmere NK, Knowlden JM, Gee JMW, Harper ME, Robertson JF, Morgan BP, Nicholson RI. Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer. Int J Cancer 2004;108:930-6.

68) Sun J, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. Cancer Res 2001;61:2276-81.

69) Yan L, Zucker S, Toole BP. Roles of the multifunctional glycoprotein, emmprin (benign; CD147), in tumor progression. Thromb Haemost 2005;93:199-204.

70) Su J, Chen X, Kanekura T. A CD147-targeting siRNA inhibits the proliferation, invasiveness, and VEGF production of human malignant melanoma cells by down-regulating glycolysis. Cancer Lett 2009;273:140-7.

71) Clynes RA, Towers TL, Presta LG, Ravetch JF. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med 2000;6:443-6.

72) Di Gaetano N, Cittera E, Nota R, Vecchi A, Grieco V, Scanziani E, Botto M, Introna M,

Golay J. Complement activation determines the therapeutic activity of rituximab in vivo. J Immunol 2003;171:1581-7.

73) Burgess AW, Cho HS, Eigenbrot C, Ferguson KM, Garrett TPJ, Leahy DJ, Lemmon MA, Sliwkowski MX, Ward CW, Yokoyama S. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. Mol Cell 2003;12:541-52.

74) Li S, Schmitz KR, Jeffry PD, Wiltzius JJW, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 2005;7:301-11.

75) Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005;5:341-53.

76) Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes J, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR. K-ras mutation and benefit from cetuximab in advanced colorectal cancer. N Eng J Med 2008;359:1757-65.

77) Cho HS, Mason K, Ramyar KX, Stanley AM, Gabell SB, Denney DW, Leahy DJ. Structure of the extracellular region of HER2 alone and in complex with Herceptin Fab. Nature 2003;421:756-60.

78) Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Prac Oncology 2006;3:269-80.

79) Franklin MC, Carey KD, Vajdas FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell 2004;5:317-28.

80) Pohl M, Stricker I, Schoeneck A, Schulmann K, Klein-Scory S, Schwarte-Waldhoff I, Hasmann M, Tannapfel A, Schmiegel W, Reinacher-Schick A. Antitumor activity of the HER2 dimerization inhibitor pertuzumab on human colon cancer cells in vitro and in vivo. J Cancer Res Clin Oncol 2009 published online.

81) Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.