Oesophageal Cancer – Novel Targets for Therapy

With focus on Hsp90, EGFR, LRIG, microtubule and telomerase

XUPING WU
Dissertation presented at Uppsala University to be publicly examined in Auditorium Minus, Gustavianum, Akademigatan 3, 75310, Uppsala, Tuesday, June 14, 2011 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Oesophageal cancer is a malignant and aggressive disease with very poor survival. The aim of this thesis was to evaluate novel therapeutic targets in oesophageal cancer.

In paper I, Hsp90 was expressed in all 81 oesophageal cancer tissues and also in nine oesophageal cancer cell lines. A specific Hsp90 inhibitor, 17-AAG, could efficiently inhibit cell proliferation, cell survival and sensitize oesophageal cancer cells to gamma photon irradiation. By inhibition of Hsp90 using 17-AAG, EGFR- and IGF-1R-mediated signalling was downregulated.

In paper II, tumour samples from 80 oesophageal cancer patients were investigated for the expression of EGFR and LRIG1-3. Based on a total score of intensity and expression fraction a trend towards survival differences was found for LRIG2 (p=0.18) and EGFR (p=0.09). Correlation analysis revealed a correlation between expression of EGFR and LRIG3 (p=0.0007). Significant correlations were found between LRIG1 mRNA expression levels and sensitivity to cisplatin (r = - 0.74), docetaxel (r = - 0.69), and vinorelbine (r = - 0.82).

In paper III, microtubule targeting drugs podophyllotoxin (PPT), vincristine and docetaxel inhibited survival and proliferation of oesophageal cancer cells. Unexpectedly, experiments showed that microtubule destabilising agents inhibited EGFR phosphorylation and signalling. A tyrosine phosphatase inhibitor, sodium orthovanadate, was able to reverse the EGFR dephosphorylation.

In paper IV, imetelstat, a telomerase antagonist, inhibited telomerase activity, colony formation ability and decreased proliferation of oesophageal cancer cells. Inhibition of telomerase activity by imetelstat led to an increase of 53BP1 foci indicating induction of DSBs. Furthermore, the fraction and size of radiation-induced 53BP1 foci were increased by imetelstat pre-treatment.

In conclusion, Hsp90 and telomerase represent potential therapeutic targets in oesophageal cancer. And, the implication of EGFR and LRIG as prognostic factors is limited. Furthermore, disruption of the microtubule network may activate a protein tyrosine phosphatase that can regulate EGFR phosphorylation.

Keywords: Hsp90, EGFR, LRIG, microtubule, telomerase, oesophageal cancer, imetelstat, DNA double-strand break, 17-AAG, radiation, prognosis, radiosensitisation, microtubule targeting agents

Xuping Wu, Department of Radiology, Oncology and Radiation Science, Oncology, Akademiska sjukhuset, Uppsala University, SE-751 85 Uppsala, Sweden.

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urn:nbn:se:uu:diva-152614 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-152614)
This book is dedicated to Lina and Ruiyan
When I am writing this book, I am thinking Ruiyang when you can read your old man’s book? 😊

Finally! But a new start always followed
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

17-AAG                          17-Allylamino-17-demethoxygeldanamycin
ADP Adenosine diphosphate
Akt Protein kinase B
ALT Alternative lengthening of telomeres
ATP Adenosine triphosphate
bp base pair
53BP1 p53 binding protein
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DNA-PK DNA protein kinase
DSB Double strand break
EGFR (HER1, ErbB1) Epidermal growth factor receptor
Erk1/2 Extracellular signal regulated kinases
FMCA Fluorometric microculture cytotoxicity assay
5-FU 5-fluorouracil
GDP Guanosine diphosphate
GTP Guanosine triphosphate
Gy Gray (unit of absorbed radiation dose)
γH2AX Histone H2AX phosphorylated on serine 139
HDAC6 Histone deacetylase 6
HER2 (ErbB2, Neu) Human epidermal growth factor receptor 2
Hsp90 Heat shock protein 90
hTERT Catalytic subunit of telomerase
hTR RNA component of telomerase
IGF-1R Insulin-like growth factor -1 receptor
LRIG Leucine-rich repeats and immunoglobulin-like domains
MAPK Mitogen-activated protein kinases
MVBs Multivesicular bodies
NQO1 NAD(P)H:quinone oxidoreductase 1
p53 protein 53
PCR Polymerase chain reaction
PPT Podophyllotoxin
PTP Protein tyrosine phosphatase
RNA Ribonucleic acid
RT-PCR Real time polymerase chain reaction
SER Sensitiser enhancement ratio
siRNA Small interfering RNA
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>shRNA</td>
<td>Small hairpin RNA or short hairpin RNA</td>
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<td>TRAP</td>
<td>Telomeric repeat amplification protocol</td>
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Introduction

Oesophageal cancer

Oesophageal carcinoma is the sixth most common cause of cancer-related death and a total of 462,000 new cases were diagnosed during 2002 (Devesa et al, 1998; Parkin et al, 2005). The dominant presenting symptom of oesophageal carcinoma is dysphagia, which is present in approximately 70% of all patients at diagnosis (Enzinger & Mayer, 2003). Epithelial-like tumours are classified as squamous cell carcinoma whereas glandular-like tumours are classified as adenocarcinoma. Squamous cell carcinoma and adenocarcinoma are the two dominating histologies in oesophageal carcinoma patients. Survival rates are poor for patients with oesophageal carcinoma. A contributing factor to the poor prognosis is the fact that the majority of patients do not seek medical attention until the tumour has already gained substantial volume or even disseminated (Talback et al, 2003). Treatment of oesophageal cancer is divided into curatively and palliatively intended treatment. The goals of palliative treatment in oesophageal carcinoma are maintenance of nutrition, relief of dysphagia as well as improving quality of life for terminal cancer patients. Strategies for palliative treatment include brachytherapy, external radiotherapy, chemotherapeutic drugs, oesophageal dilatation, and oesophageal stenting. Surgery, offered as curatively intended treatment, is the first choice of treatment for patients in good medical condition and loco-regional disease. The development of new surgical techniques and better postoperative care has improved prognosis for patients undergoing surgery as the sole treatment, with 2-year survival rates ranging from 35 to 42 percent, and 5-year survival rates from 15 to 24 percent (Enzinger & Mayer, 2003). Curatively intended radiation therapy can be delivered to locoregional tumours as conventional external radiation alone or in combination with intra-luminal brachytherapy. External radiation is usually administered in fractions of 1.8-2.0 Gray (Gy) five times a week until the final dose of 40-70 Gy is reached. The conventional chemotherapy for oesophageal carcinoma includes cisplatin and 5-fluorouracil (5-FU) as a standard cytotoxic drug combination. The response rate for cisplatin as a single agent is approximately 20% (Panettiere et al, 1984; Ravry et al, 1985), whereas the combination of cisplatin and 5-FU demonstrates a response rate ranging from 35%-65% (Bleiberg et al, 1997; Wadler et al, 1996). Other combinations with cisplatin have also been studied, including paclitaxel and irinotecan, with a response rate of 40% and 36%, respectively (Ilson, 2004; Polee et al, 2002).
The combination of radiotherapy and concurrent chemotherapy with cisplatin and fluorouracil has led to long-term survival in approximately 25% of patients, an outcome similar to that associated with surgery alone. In a recent meta-analysis of survival benefits from neoadjuvant chemoradiotherapy or chemotherapy in oesophageal carcinoma (Gebski et al, 2007), Gebski et al. demonstrated a significant survival benefit for preoperative chemoradiotherapy and, to a lesser extent, for chemotherapy in patients with adenocarcinoma of the oesophagus. However, patients with advanced metastatic disease that are treated with palliative chemotherapy have a median survival of less than one year, and the 5-year survival rate of all diagnosed patients is only around 15%. Thus, there is a need for novel strategies to improve current therapy. With the recent advances in drug development, there are emerging possibilities to utilise antagonists of growth factor signal transduction pathways in targeted therapy. This work focuses on possible emerging treatment strategies including novel targets for therapy.

The role of EGFR in oesophageal cancer

The human epidermal growth factor receptor (HER) family of growth factor receptors is comprised of four members, i.e. EGFR (HER1, ErbB1), HER2 (ErbB2, Neu), HER3 (ErbB3) and HER4 (ErbB4) (Citri & Yarden, 2006). These receptors are tyrosine kinases that are activated by ligand-induced dimerisation. There are several ligands for the receptors in the HER family, and these have different binding specificities, resulting in formation of homo- or heterodimeric receptor complexes. Interestingly, HER2 does not have a ligand but is utilised as a heterodimeric partner by the other receptors. However, overexpression of HER2 may induce formation of homodimers, and in fact HER2 has oncogenic properties when overexpressed. HER family members are commonly activated in several cancers (Normanno N, 2005) by autocrine stimulation, by mutations, or by overexpression. A consequence of unregulated and improper receptor activation is induction of signals that promote proliferation, survival, migration and angiogenesis - events that are all central for tumour development and progression.

EGFR is often overexpressed in oesophageal cancers, both squamous cell carcinoma and adenocarcinoma, which in certain cases also express the ligands EGF or transforming growth factor α (TGFα), establishing autocrine growth promoting loops (Chen et al, 1991; Helmut et al, 1999; Jankowski et al, 1993; Jones GJ, 1993; Mukaida H, 1991; Mukaida H, 1990). In fact, EGFR overexpression has been demonstrated to correlate with poor prognosis (Gibson et al, 2003; Kitagawa et al, 1996; Mukaida H, 1991; Ozawa et al, 1989; Yoshida et al, 1993). However, not all studies have found such a connection (Friess et al, 1999). Although EGFR is commonly expressed, mutations in EGFR are not frequently found in oesophageal cancers
(Puhringer-Oppermann et al, 2007; Sudo et al, 2007). However, a mutation in the kinase domain of EGFR, S768I, which confers increased sensitivity towards gefitinib has been found in the oesophageal cancer cell line Kyse450 as well as in primary oesophageal carcinoma (Guo et al, 2006b).

![Diagram of EGFR and downstream signalling](image)

**Figure 1.** Activation of EGFR and downstream signalling.

As shown in figure 1, activation of EGFR signalling has been implicated in survival and proliferation mainly by activating downstream signalling mitogen-activated protein (MAP) kinases and protein kinase B (Akt). Activation of EGFR can also enhance metastasis via modulation of cell adhesion, angiogenesis, invasion and migration. For example, it has been observed in some, but not all, oesophageal tumour cell lines that activation of EGFR may increase the expression of matrix metalloproteases (MMPs), which are important for the degradation of the extracellular matrix that is necessary for tumour invasion and metastasis (Xu et al, 2003; Yoshida et al, 1990). Moreover, in the oesophageal cancer cell line TE-2R, it was shown that EGF induces re-localisation of E-cadherin from the lateral adhesion sites to a more uniform distribution over the cell surface which was correlated with changed cell morphology and increased invasiveness (Shiozaki et al, 1995).

Strategies attempting to therapeutically target the EGFR include antibodies binding to the extracellular domain, such as cetuximab, or low molecular weight inhibitors blocking the kinase activity, such as gefitinib (Iressa, ZD 1839) and erlotinib (Tarceva, OSI-774). Gefitinib has been shown to induce growth arrest of human oesophageal cancer cell lines (Hara et al, 2005; Teraishi et al, 2005). Teraishi et al found no correlation between the re-
response to gefitinib and the level of EGFR expression (Teraishi et al., 2005), but in gefitinib-responsive cells they observed a dose-dependent increase of cell cycle arrest at G1 phase. Moreover, the anti-tumour effect of the death receptor ligand tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) was enhanced by gefitinib treatment, even in cells considered to be TRAIL resistant (Teraishi et al., 2005). The mechanism for this was shown to involve inhibition of EGFR-mediated Akt phosphorylation by gefitinib, leading to Bcl-xL inactivation and caspase-9 activation, thereby promoting apoptosis of cells through a mitochondrial-dependent apoptotic pathway. Importantly, gefitinib has been demonstrated to increase the effect of radiotherapy; the mechanism for this is believed to be inhibition of the Akt and Erk survival pathways downstream of the EGFR and subsequent sensitisation of the cell to radiation-induced apoptosis (Taira et al., 2006). Several clinical trials have studied EGFR targeting in oesophageal cancer. In a phase II trial by using gefitinib, Ferry et al. reported that gefitinib is an active treatment in advanced adenocarcinoma of oesophagus with a disease control rate (PR; partial response or SD; stable disease) of 58 % (Ferry et al., 2004). In a clinical trial with erlotinib, Tew et al. reported a disease control rate of 54.5 % for a cohort of 22 patients with metastatic, pre-treated oesophageal squamous cell carcinoma or adenocarcinoma (Tew W, 2005).

EGFR and the microtubule system

Microtubule structure and inhibition

The cytoskeleton is comprised of actin filaments, intermediate filaments and microtubules. Microtubules are dynamic structures constantly undergoing polymerisation and depolymerisation. Microtubule dynamics are important for the development and maintenance of cell shape, signal trafficking, and cell mitosis and division. The functional diversity of microtubules is achieved in several different ways; by several post-translational modifications, binding with microtubule-associated proteins and expression of different tubulin isotypes in specific cells. Microtubules play a crucial role in the process of mitosis, which separates the duplicated chromosomes into two daughter cells before the cleavage of the cells. Thus, microtubule and its dynamics are important targets for anticancer drugs. Several drugs that intervene with microtubule structure and dynamics are in clinical use. One group is the taxanes including docetaxel and paclitaxel which bind to polymerised microtubules and stabilise the GDP-bound form of β-tubulin by forcing them into a configuration resembling the GTP-bound state (Burkhart et al., 1994). Another group is vinca alkaloids including vincristine and vinorelbine that bind closely to the GTP-binding region in β-tubulin and destabilise microtubules (Lobert et al., 1996).
As shown in figure 2, α- and β-tubulin heterodimers polymerise into a hollow tube denoted microtubule. The polymerisation dynamics of the microtubule is regulated by GTP hydrolysis; a microtubule end containing tubulin-bound GTP is stable and prevents depolymerisation, while hydrolysis of tubulin-bound GTP to GDP induces conformational changes and destabilises the microtubule polymer, although within the microtubule GTP can be hydrolysed to GDP (Jordan & Wilson, 2004; Li et al., 2002). The biological functions of microtubules in cells are determined and regulated mainly by their polymerisation dynamics. Microtubules show two kinds of polymerisation dynamics behaviour, “dynamic instability” and “treadmilling”. During “dynamic instability” the microtubule plus end grows or shortens greater than the minus end (Mitchison & Kirschner, 1984), whereas “treadmilling” behaviour means that the microtubule plus end grows while the minus end shortens without changing the microtubule net length (Margolis & Wilson, 1978; Rodionov & Borisy, 1997). Treadmilling is created by different subunit concentrations at the opposite ends. Dynamic instability behaviour is prominent in cells but compatible with treadmilling behaviour.

EGFR trafficking

Activated EGFR is internalized through clathrin-dependent endocytosis. The endocytosed activated EGFR is destined either to be transported to recycling endosomes and return to the cytoplasmic membrane or be transported to multivesicular bodies (MVBS) and then further to lysosomes for degradation. Microtubules are involved in EGFR endocytic trafficking and the movement of EGFR containing endosomes along microtubules needs microtubule-associated dynein motors (Aniento et al., 1993; Driskell et al., 2007). Gao et al. reported that histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase binding to dynein motors, associates with the endosomal compartments and controls EGFR trafficking and degradation (Gao et al.). This is consistent with data from Deribe et al. showing that HDAC6 negatively regulates EGFR endocytosis and degradation by controlling the acetylation status of α-tubulin and subsequently receptor trafficking along microtubules (Lissanu Deribe et al., 2009).
Figure 2. A microtubule is composed of α-tubulin and β-tubulin heterodimers. Each microtubule has a plus (+) end and a minus (-) end. Both ends can grow or shorten during microtubule dynamics. Modified from Jordan (Jordan & Wilson, 2004).

Hsp90 inhibition in cancer

Hsp90 function and inhibition

Heat shock protein 90 (Hsp90) is an abundant protein that functions as a chaperone, thus preventing protein aggregation and helping denatured proteins to refold in an ATP-dependent fashion. Hsp90 has a weak intrinsic ATPase activity, which can be enhanced by interactions with co-chaperones and client proteins. The Hsp90 super-chaperone cycles between an open and a closed state. As shown in figure 3, replacement of ADP by ATP in Hsp90 alters its conformation leading to the recruitment of co-chaperones, including p23, certain immunophilins and cdc37, which fold and stabilise client proteins in a state that can bind ligands or respond to stimuli. However, the Hsp90 super-chaperone machine is never static. If the client proteins fail to bind the ligands, the chaperone cycle will reverse. Upon ATP hydrolysis, Hsp90 releases the co-chaperones that associate with the ATP-bound state and recruits co-chaperones, like Hsp40, Hsp70, Hip and Hop, to the ADP-
bound conformation. When client proteins are associated with the ADP-bound Hsp90 conformation, they are no longer folded properly and targeted for degradation by the proteasome. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is an inhibitor of Hsp90 that binds to the nucleotide binding site with an affinity much greater than either ATP or ADP. The 17-AAG-bound conformation mimics the chaperone’s ADP-bound conformation and thereby promotes release of the client proteins which then may be degraded via the ubiquitin-proteasome pathway (Isaacs et al., 2003). It has been shown that the E3 ubiquitin ligase contains chaperone-interacting motifs. Xu et al. reported that the carboxyl terminus of HSC70-interacting protein (CHIP) is a U-box-containing E3 ubiquitin ligase that binds to Hsp90 and Hsp70 by means of its tetratricopeptide (TPR) motif to facilitate the ubiquitination of Hsp90 client proteins like ErbB2 (Xu et al., 2002).

Figure 3. Nucleotide-dependent cycling of the Hsp90 super-chaperone machine. Cycling of the Hsp90 machine is driven by ATP-hydrolysis which is regulated by co-chaperones and enhanced by client protein binding. Co-chaperones, including cdc37, p23, immunophilins, cyclophilin 40 as well as phosphatase pp5, Hsp40, Hip, BAG-1 Hsp70 and Hop, can associate with different conformations.
The list of Hsp90 client proteins has expanded during years of research, as shown in table 1. In many cancer cells, Hsp90 functions to protect and stabilise overexpressed or mutated signal transduction proteins, thus indirectly promoting cell growth and survival (Citri et al, 2004; Zhang & Burrows, 2004). Inhibition of Hsp90 leads to degradation of client proteins, which provides an efficient strategy to target cancer. For example, L858R and exon 19 deletions in the kinase domain of ErbB1 confer sensitivity to gefitinib and erlotinib in non-small cell lung cancer, however a second mutation like T790M leads to acquired resistance to these tyrosine kinase inhibitors. Shimamura et al. reported that mutated ErbB1 is associated with Hsp90 and inhibition of Hsp90 results in a much quicker degradation of mutant ErbB1 compared to wild-type ErbB1 (Shimamura et al, 2005). Around 30 to 40% of ErbB2-positive breast tumours are trastuzumab-resistant. Zsebik et al. showed that the Hsp90 inhibitor 17-AAG reduces ErbB2 and inhibits proliferation of transtuzumab-resistant ErbB2-overexpressing breast tumour cell lines (Zsebik et al, 2006). It has been noticed that in certain cancer cells the function of Hsp90 is more critical than for normal cells (Bisht et al, 2003; Ferrarini et al, 1992). Hence, there is a possibility that inhibition of Hsp90 could sensitize tumour cells to cytotoxic therapy while sparing normal cells. Indeed, in some cases such sensitisation has been observed and 17-AAG is currently in phase II single agent trials and in phase I/II combination trails (Georgakis & Younes, 2005).
Table 1. Hsp90 client proteins associate with cancer development. Based on data from reviews by Ekman et al. and Zhang et al. (Ekman et al., 2010; Zhang & Burrows, 2004).

<table>
<thead>
<tr>
<th>Kinases</th>
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<tr>
<td>Akt</td>
</tr>
<tr>
<td>Bcr-Abl</td>
</tr>
<tr>
<td>Cdk 1, 2, 4, 6, 9, 11</td>
</tr>
<tr>
<td>ErbB2</td>
</tr>
<tr>
<td>Mutant ErbB1</td>
</tr>
<tr>
<td>c-MET</td>
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<tr>
<td>IGF-1R</td>
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<tr>
<td>MEK</td>
</tr>
<tr>
<td>Raf-1</td>
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<tr>
<td>Mutated B-Raf</td>
</tr>
<tr>
<td>c-Kit</td>
</tr>
<tr>
<td>VEGFR1</td>
</tr>
<tr>
<td>VEGFR2</td>
</tr>
<tr>
<td>FAK</td>
</tr>
<tr>
<td>pp60v-Src, c-Src</td>
</tr>
<tr>
<td>PDK1</td>
</tr>
<tr>
<td>Src related tyrosine kinases: Yes, Fps, Fes, Fgr, and Lck</td>
</tr>
<tr>
<td>IxB Kinase α and β</td>
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<td>Flt3</td>
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<tr>
<th>Transcription factors</th>
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<tr>
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<tr>
<td>HSF-1</td>
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<tr>
<td>p53</td>
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<tr>
<td>Stat3</td>
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<tr>
<td>Sp1</td>
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<tr>
<td>Hypoxia-inducible factor-1 and -2</td>
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<th>Others</th>
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<tr>
<td>Mdm2</td>
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<tr>
<td>Proteasome</td>
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<tr>
<td>Survivin</td>
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<tr>
<td>Telomerase</td>
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<tr>
<td>Skp2 complex</td>
</tr>
<tr>
<td>Cyclin B</td>
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<tr>
<td>Bid</td>
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<td>Bcl-XL</td>
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Radiosensitisation

Hsp90 also has a potential as a target of radiosensitisation because a number of Hsp90 client proteins, for example HER2, Akt, Raf-1 and DNA-PK, have been associated with radioresponse (Dote et al., 2006; Russell et al., 2003). It has been reported that 17-AAG enhances the radiosensitivity of cells from glioma, prostate carcinoma, cervical carcinoma, squamous lung carcinoma and we could demonstrated similar results in oesophageal cancer cells (Bisht et al., 2003; Noguchi et al., 2006; Russell et al., 2003; Wu et al., 2009). Dote et al. reported that loss of ErbB2 after Hsp90 inhibition is sufficient to reduce
ErbB1 activity resulting in radiosensitisation, while ErbB1 activity was maintained when forming ErbB1/ErbB3 heterodimers in resistant cells (Dote et al., 2005). Thus, ErbB3 predicts radiosensitisation induced by Hsp90 inhibition. It was also reported that Hsp90 inhibition results in decrease of ErbB1 activity and transportation of ErbB1 into the nucleus after radiation, which significantly diminishes the radiation-induced interaction between ErbB1 and DNA-PK, consequently leading to a reduction in DNA-PK activation compromising the repair of double strand breaks (DSBs) (Dote et al., 2006).

Hsp90 as a prognostic factor

Pick et al. reported that Hsp90 expression was associated with decreased survival in breast cancer patients and high Hsp90 expression could be an independent prognostic factor (Pick et al., 2007). Furthermore, they found that high Hsp90 expression was associated with larger tumours, higher tumour grade as well as lymph node involvement, suggesting that high Hsp90 expression may serve as a prognostic factor identifying patients in need of more aggressive therapy. In line with these data, they found that Hsp90 expression was high in breast cancer cell lines (Pick et al., 2007). However, in the work by Faried et al., Hsp90 expression failed to predict prognosis in oesophageal squamous cell carcinoma. However, Hsp60 which has pro-apoptotic properties was found to be a good prognostic indicator (Faried et al., 2004). In our study, all tumours samples displayed Hsp90 staining whereas normal tissue displayed no or weak staining, indicating that Hsp90 is selectively expressed in oesophageal cancer (Wu et al., 2009).

The development of Hsp90 inhibitors

Although 17-AAG is now in several early-stage clinical trials, it is notable that 17-AAG has poor pharmaceutical properties because of its relatively poor water solubility. It requires the addition of organic excipients like DMSO or polyoxyl castor oil (Cremophor), which can cause side effects, such as hepatotoxicity and cardiotoxicity (Sydor et al., 2006). Hence, IPI-504 which is a highly soluble hydroquinone hydrochloride derivative of 17-AAG has been developed by Infinity Pharmaceuticals, Inc. (Sydor et al., 2006). It has been shown that IPI-504 is a potent inhibitor of Hsp90 and has antitumour effects both in vivo and in vitro, with slightly more potency compared to 17-AAG.

Despite results from studies in mice demonstrating the anti-tumour activity of Hsp90 inhibitors, such as geldanamycin (GA) and 17-AAG, recent reports indicate that these inhibitors lack significant single agent clinical activity. McCollum et al. reported that P-glycoprotein high expressing cells had a IC$_{50}$ for 17-AAG that was six-fold compared with parental cells (McCollum
et al, 2008). Guo et al. showed that NAD(P)H:quinone oxidoreductase 1 (NQO1) expressing cells were twelve-fold more sensitive to 17-AAG growth inhibition compared with parental cells (Guo et al, 2005). More importantly, they found that the increased sensitivity to 17-AAG could be abolished if the cells were pre-treated with ES936 (inhibitor of NQO1). These results indicate that 17-AAG as an Hsp90 inhibitor is dependent on P-glycoprotein and NQO1 and this needs to be exploited in future clinical studies. NVP-AUY922, a novel resorcinic isoxazole amide Hsp90 inhibitor, is P-glycoprotein and NQO1 independent (Eccles et al, 2008). It was reported that NVP-AUY922 potently inhibited Hsp90 ($K_d = 1.7$ nM) and proliferation of human tumour cells with IC$_{50}$ values of approximately 2 to 40 nM. NVP-AUY922 is the most potent Hsp90 inhibitor described so far and has now entered phase I clinical trials.

LRIG proteins in cancer
Backgroud
The human leucine-rich repeats and immunoglobulin-like domains (LRIG) protein family comprises LRIG-1, -2, and -3. LRIG proteins are integral membrane proteins containing an extracellular region consisting of a leucine-rich repeats (LRR) domain and three immunoglobulin-like domains, a transmembrane domain, and a cytosolic tail (Hedman & Henriksson, 2007). LRIG1 has been reported to be a tumour suppressor and shown to counteract the signalling of EGFR (Gur et al, 2004; Laederich et al, 2004), MET (Shattuck et al, 2007) and RET (Ledda et al, 2008). LRIG1 interacts through its extracellular domain with the extracellular parts of the human ErbB family members, then recruiting cytoplasmic E3 ubiquitin ligases resulting in ErbB receptor ubiquitination, internalisation and degradation. Thus, LRIG1 participates in a negative feedback loop involving receptor ubiquitination and degradation leading to the suppression of ErbB receptor signalling. Less is known about the functions of LRIG2 and LRIG3. In vitro experiments have shown that green fluorescent protein (GFP)-tagged LRIG proteins exhibit different subcellular distribution in a cell-type specific manner. For example, LRIG proteins localise to the plasma membrane in Vero fibroblasts or U-105 MG glioma, whereas in human embryonic kidney-293 cells or GL15glioma cells they localise to the perinuclear region (Guo et al, 2006a). In vivo, LRIG proteins also have different subcellular localisations, where perinuclear localisation in astrocytic tumours predicts better patient survival and cytoplasmic expression of LRIG2 in oligodendroglial tumours predicts worse patient outcome (Guo et al, 2006a; Holmlund et al, 2009).
LRIG as a prognostic factor

LRIG transcripts and proteins are ubiquitously expressed in normal tissues and organs, like cervix, colon, heart, lung and skin etc. (Hedman & Henriksson, 2007). LRIG expression has also been suggested to be of prognostic significance in several malignancies (Hedman & Henriksson, 2007). Thomasson et al. studied 31 renal cell carcinomas for expression of the potential tumour suppressor LRIG1 as well as EGFR by quantitative real-time RT-PCR (Thomasson et al., 2003). They showed that EGFR was upregulated and LRIG1 was downregulated in conventional renal cell carcinomas. The observed downregulation of LRIG1 and increased EGFR/LRIG1 ratio is consistent with LRIG1 being a suppressor of oncogenesis in renal cell carcinomas by counteracting the tumour-promoting properties of EGFR (Thomasson et al., 2003). Tanemura et al. studied LRIG1 expression in 38 cases of cutaneous squamous cell carcinoma (SCC) by immunohistochemistry and showed that LRIG1 is an excellent prognostic indicator for cutaneous SCC (Tanemura et al., 2005). LRIG1 was highly expressed in well-differentiated lesions of cutaneous SCC and significantly inversely correlated with metastatic rate. Furthermore, patients with high LRIG1 expression had a survival benefit (p = 0.03) compared to the group with low LRIG1 expression (Tanemura et al., 2005). Holmlund et al. studied LRIG expression in 63 oligodendroglial tumours by using immunohistochemistry and found that cytoplasmic LRIG2 is an independent prognostic factor associated with poor patient survival, suggesting that LRIG2 may have a function different from that of LRIG1 (Holmlund et al., 2009). LRIG1 expression has been associated with better survival in early-stage uterine cervical carcinoma (Lindstrom et al., 2008). However, Hedman et al. reported that high expression of LRIG2 was associated with poor survival in early-stage uterine cervical squamous cell carcinoma. A combination of high LRIG2 expression and low LRIG1 expression identified in women with uterine cervical carcinoma showed a very poor prognosis, the 10-year survival rate was only 26% compared to 66% for the remaining study population (Hedman et al., 2010). Regarding LRIG3, Guo et al. reported that peri-nuclear staining of LRIG1-3 was associated with better survival and lower WHO grade in astrocytic tumours, where LRIG3 as an independent prognostic factor was associated with a lower proliferation index; within the groups of grade III and grade IV astrocytic tumours peri-nuclear staining of LRIG3 significantly correlated with better survival (Guo et al., 2006a).

Telomerase

Background

Telomerase is a reverse transcriptase involved in the unlimited growth of tumour cells. It elongates telomeric DNA by adding TTAGGGG repeats to the
chromosome ends, thus compensating for telomere shortening during the DNA replication cycle (Lingner et al., 1997). Telomerase is a complex multi-subunit ribonucleoprotein enzyme in which the minimal catalytic core includes the reverse transcriptase protein subunit hTERT and the telomerase RNA subunit (hTR). hTR functions as the template for the enzyme to add the telomere repeats at the single-strand telomere end (Artandi & DePinho, 2009). Human telomeres are initially composed of 15 to 20 kbp of TTAGGG double-strand DNA repeats followed by a single strand 3’-G-rich overhang which is inserted into the double-strand DNA, thereby creating a ring structure of the T-loop and a smaller single-strand D-loop (Griffith et al., 1999). A variety of proteins are associated with telomeres. The shelterin complex, composed of TRF1, TRF2 (telomeric repeat binding proteins) and POT1 (protection of telomeres 1), TIN2, Rap1 and TPP1, are telomere-specific and bind to telomeres, having fundamental roles in allowing cells to distinguish the telomeres from DNA-damage, telomere length regulation, preservation of telomeres and regulating binding of telomerase to telomeres (de Lange, 2005).

The telomere hypothesis suggests that telomere shortening is the fundamental mitotic clock in normal human somatic cells limiting their replicative potentials. When average telomere DNA reaches a critically short length, cell division is arrested irreversibly (Harley et al., 1990). As shown in figure 4, the process of normal cells transforming to immortalized cancer cells is strictly regulated by telomeres length. Most differentiating somatic cells never reach telomere length-dependent senescence and they are eliminated with normal turnover; new cells will descend from stem cells. However, a fraction of cells can enter replicative senescence showing cell cycle arrest and low metabolic rate with critical telomere length around 5 kbp. If the tumour suppressor gene p53 is mutated and inactivated, cells can escape senescence and undergo further divisions for about 20 doublings until the cells reach a stage of crisis. At the crisis stage the telomeres are very short, about 1 to 3 kbp, resulting in genomic instability and cell death. A rare fraction of cells can pass this checkpoint by activation of telomerase to stabilise the telomere length and become immortalised (Ducray et al., 1999). Telomerase activation is one option that immortalised cells use to maintain telomere length. A fraction of cells can become immortalised and maintain the telomere length by using the alternative lengthening of telomeres (ALT) mechanism. The mechanism of telomere elongation of ALT is dependent on DNA-homologous recombination and the process is not fully apprehended (Cesare & Reddel, 2010). About 10% of human tumours use ALT to maintain telomere length without expression of telomerase activity (Bryan et al., 1997; Cesare & Reddel, 2008). However, Seger et al. reported that normal human cells can be transformed to cancer cells, capable of forming tumours in immuno-compromised mice by the combined expression of E1A, Ha-RasV12 and MDM12 oncogenes. The transformation did not require telom-
erase activation or an alternative telomere maintenance strategy (Seger et al, 2002).

![Telomere length diagram](image)

**Figure 4.** A normal cell can be transformed to an immortalized cancer cell by bypassing stages of senescence and crisis. The cancer cells have in general shorter telomeres compared to normal cells. Modified from Harley (Harley, 2008).

**Targeting telomerase**

Telomerase is active in the majority (80% to 90%) of tumours from all cancer types but not in normal somatic cells, and some studies have suggested that cancer stem cells are also telomerase positive (Armanios & Greider, 2005; Kim et al, 1994; Phatak et al, 2007; Shay & Bacchetti, 1997). The key advantages of targeting telomerase in comparison with most other cancer therapies are that telomerase is relatively widely and specifically expressed in cancer cells, including putative cancer stem cells. The difference between cancer and normal cells makes cancer cells more sensitivity to telomerase inhibitors, which provides a substantial therapeutic window for telomerase inhibition-based treatment. During recent years several telomerase-based anticancer approaches have been studied, as shown in figure 5. The most direct approach is telomerase inhibition by targeting telomerase reverse tran-
scriptase (hTERT) or telomerase RNA component (hTR) directly, the oligonucleotide drug imetelstat being an example of this. Imetelstat contains a 13-mer oligonucleotide thio-phosphoroamidate and a lipid group (Herbert et al, 2005). The presence of the covalently conjugated lipid group provides for high cellular uptake and bio-availability. Furthermore, the thio-phosphoroamidate internucleotide linkages in imetelstat provides high thermodynamic stability of its complex with the targeted hTR and a long half-life (Dikmen et al, 2009; Herbert et al, 2005). Imetelstat binds to the complementary telomerase RNA component (hTR) sequence in the active site region of telomerase, which leads to telomerase inhibition and telomere shortening resulting in cell cycle arrest and cell death (Asai et al, 2003). Imetelstat has been shown to cause telomere length dependent effects in a variety of tumours in vitro, for example lung cancer (Dikmen et al, 2005), breast cancer (Gellert et al, 2006), bladder cancer (Dikmen et al, 2008), myeloma (Shammas et al, 2008a), Barrett’s adenocarcinoma (Shammas et al, 2008b) and hepatoma (Djojosubroto et al, 2005). Furthermore, imetelstat has been reported to exert growth inhibiting effect on putative cancer stem cells. Long-term treatment with imetelstat led to telomere shortening, cell proliferation arrest and eventually death in glioblastoma initiating cells in vitro (Marian et al, 2010). Imetelstat can cross the blood brain barrier causing inhibition of telomerase activity and a marked decrease of tumour growth in glioblastoma xenografts after systemic administration (Marian et al, 2010). Joseph et al. also reported that imetelstat can deplete cancer stem cells in breast and pancreatic cancer cell lines (Joseph et al, 2010). Imetelstat has now entered clinical trials for multiple tumour types, including chronic lymphocytic leukaemia, multiple myeloma, lung, breast and advanced tumours, as a single agent or combined with other chemotherapeutic agents (Harley, 2008).

hTERT-based immunotherapy is another efficient approach to target telomerase and several products are in clinical trials (Domchek et al, 2007; Nava-Parada & Emens, 2007; Su et al, 2005). Telomerase-active immunotherapy is designed to stimulate the immune system to attack and kill telomerase positive cells that express hTERT. The immune system of the patients can be activated by exposing antigen-presenting cells to large amounts of hTERT peptide, mRNA or plasmid DNA in vivo. Other telomerase-targeting therapy approaches include telomere disrupting agents, agents that block telomerase expression or biogenesis and hTERT promoter-driven suicide gene therapy. The telomere disrupting agents alter the structure of telomeres to prevent the binding to telomerase. For example, small molecules stabilise the G-quadruplex structure of telomeres leading to inhibition of telomerase activity or telomere uncapping (Gowan et al, 2002; Salvati et al, 2007). To block telomerase expression or biogenesis, agents such as Hsp90 inhibitors or siRNA targeting hTERT and hTR can be used (Li et al, 2005). For hTERT promoter-driven suicide gene therapy, a designed toxic gene is placed down-
stream of promoter region of hTERT (Bilsland et al., 2003). In cancer cells, hTERT expression accompanied with suicide gene expression can kill cancer cells while sparing normal cells.

![Diagram of telomerase and related terms]

**Figure 5.** Five approaches to target telomerase in cancer cells.

**Telomeres and ionising radiation**

Most of the human somatic cells undergo senescence after a limited number of cell population doublings, which can be triggered when telomeres cannot protect the end of chromosomes. Loss of telomerase activity results in critical telomere shortening, leading to genetic instability, senescence or apoptosis through DNA-damage signals. It has been reported that senescence reflects a DNA-damage checkpoint response with activation of damage checkpoint kinases CHK1 and CHK2 and nuclear γH2AX foci, which is directly connected to uncapped telomeres at chromosome ends (d'Adda di Fagagna et al., 2003). The role of telomere length in DNA-damage repair and radiosensitivity of cancer cells has been reported. Gomez-Millan et al. reported that long-term treatment with imetelstat led to progressive reduction in telomere length and sensitised breast cancer cells to radiation in a survival colony formation assay (Gomez-Millan et al., 2007). In an in vivo study, Wong et al. used the telomerase-deficient mice null for the essential telomerase RNA gene, showing that the loss of telomerase activity at an early stage had no discernable impact compared to wild-type. However, later generations showed a critically short telomere length, apoptosis and proliferative defects in many organs as well as a radiosensitivity syndrome. On a cellular level, primary cells of gastrointestinal and embryonic origin showed increased apoptosis, increased radiosensitivity and delayed DNA-repair kinet-
ics. (Wong et al., 2000). Cells can also respond to ionising radiation in a telomere length independent but telomerase-dependent way. Telomerase has during recent years been assigned to exert pro-survival functions, unrelated to telomere length maintenance. Masutomi et al. reported that suppression of the telomerase catalytic subunit (hTERT) expression by using hTERT shRNA abrogates the cellular response like H2AX phosphorylation, NBS-1 foci accumulation, phosphorylation of BRCA1 and up-regulation of p53 after treatment with ionising radiation or irinotecan and etoposide which induce DNA DSBs (Masutomi et al., 2005). Furthermore, cells lacking hTERT exhibited increased radiosensitivity, reduced capacity for DNA-repair and fragmented chromosomes (Masutomi et al., 2005). The hTERT suppression leads to impaired genomic stability by decreasing levels of histone H3-lysine (K) 9 dimethylation and increasing amounts of H3-K9 acetylation as well as decreasing H4-K12 acetylation (Masutomi et al., 2005), which may explain impaired DNA repair capacity in cells lacking hTERT. This is partially proved by Rum et al., reporting that radiation up-regulates telomerase activity and also promotes the translocation of telomerase from the cytoplasm into the nucleus via activation of the Ras/Phosphatidylinositol 3-kinase/Akt pathway (Ram et al., 2009). This transient up-regulation of telomerase activity may confer a stress response in cells and protect them from DNA damage.
Aims

I To evaluate prognostic value of Hsp90 expression in oesophageal carcinoma and the potential of Hsp90 as the primary target for novel therapy using 17-AAG in oesophageal cancer.

II To investigate the clinical implications of EGFR and LRIG1-3 expression as clinical prognostic factors in oesophageal carcinoma and the importance of EGFR and LRIG mRNA levels for the sensitivity of standard chemotherapeutic agents in oesophageal carcinoma cells.

III EGFR is widely expressed in oesophageal carcinoma and oesophageal cancer cells. The aim was to investigate the sensitivity of oesophageal cancer cells to microtubule targeting drugs as well as possible effects on EGFR.

IV The aim of this study was to evaluate the potential of telomerase as a target for therapy in oesophageal carcinoma by using the telomerase antagonist imetelstat in vitro.
Materials and Methods

Cell lines
Kyse30, 70, 140, 150, 180, 410, 450, 510 and 520 cell lines derived from oesophageal squamous cell carcinoma were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in a 37°C incubator with humidified atmosphere and 5% CO₂.

Patient materials
Between 1990 and 2000, 126 patients were recorded as having received treatment for oesophageal carcinoma at the Department of Oncology, Uppsala University Hospital. Treatment strategies for these patients were curatively intended radiation treatment alone, preoperative chemotherapy and radiation treatment followed by surgery or palliative treatment including radiation treatment or chemotherapy. The following clinical parameters were evaluated in patients: gender; age; weight loss; performance status at first admittance; smoking history; tumour histology; and tumour stage at first admittance, defined as localised or metastatic disease. Tumour localisation was grouped into upper (15–24 cm), middle (25–34 cm), and lower (35–46 cm) part of oesophagus. No data were available concerning surgical resection grade or surgically related complications. Collection of the clinical parameters started when the patient was first admitted to the Oncology Department. The patients were followed until March 12, 2003 and mean survival in the material was 547 days (median = 266 days, min = 5 days, max = 3675 days). The study was reviewed and approved by the research ethics committee, Uppsala University, Uppsala, Sweden.

Immunohistochemical analysis
Paper I
Hsp90 immunohistochemical (IHC) staining was performed in 81 paraffin-embedded tumour samples. HeLa cells were used as a positive control. Sections were incubated with anti-Hsp90 primary antibody and an automated
immunohistochemical system from Ventana (Benchmark; Ventana Medical Systems) was employed according to the manufacturer’s recommendations. The extent of positive tumour cells was scored using a three-grade scale: 1: < 25% positive tumour cells, 2: 25-75% positive cells, 3: > 75% of tumour cells staining positively. The intensity of immunoreactivity in tumour cells was evaluated using a four-grade scale: negative (1), weak (2) moderate (3) strong (4). The subcellular localisation was also evaluated: membranous, cytoplasmic or nuclear positivity.

Paper II

Immunohistochemical analysis of EGFR and LRIG1–3 was performed in 80 formalin-fixed and paraffin-embedded tumour samples. Briefly, 4 µm-thick tissue sections were stained with anti-EGFR, anti-LRIG1, anti-LRIG2 and anti-LRIG3 primary antibodies at the designed concentrations. Immunodetection was achieved by using horseradish peroxidase-conjugated secondary antibodies and the substrate diaminobenzidine. The slides were then counterstained with haematoxylin and mounted in glycerol-gelatin. The evaluation of the immunostaining was performed by two independent observers. Positive immunohistochemical staining in tumour cells was evaluated based on fraction of positive tumour cells and the intensity of positive staining. From these scoring data, three categories were defined: Grade 3: Strong immunoreactivity in >25% of tumour cells; Grade 2: moderate immunoreactivity in >25% of tumour cells, or strong immunoreactivity in <25% of tumour cells; Grade 1: weak immunoreactivity in >25% of tumour cells, or moderate immunoreactivity in <25% of tumour cells; Grade 0: Lack of immunostaining (negative), or weak immunostaining in <25% of tumour cells. A total score based on low fraction and intensity (graded as 0 or 1) versus high fraction and intensity (graded 2 or 3) was created for each tumour sample. The subcellular localisation of the stainings was found to be predominantly cytoplasmic.

Cytotoxicity assay

In paper II, fluorometric microculture cytotoxicity assay (FMCA) (Nygren P, 1994) was used to investigate the cytotoxic effect of seven clinically used cytotoxic drugs: 5-fluorouracil (5-FU), cisplatin, docetaxel, gemcitabine, etoposide, melphalan, and vinorelbine in nine oesophageal cancer cell lines. The FMCA is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by living cells with intact plasma membrane. The resulting fluorescence is proportional to the number of living cells. Cell survival is presented as survival index (SI % of untreated control) (shown in figure 6). The procedure of FMCA was described in figure 6. Briefly, 384-well microtiter plates were prepared with drug solutions in duplicate at 10 times the desired final drug concentration.
Cells were seeded into the drug-prepared microtiter plates at a cell density of $1 \times 10^5$ cells/ml and incubated for 72 h at 37 °C in a humidified 5% CO$_2$ atmosphere. FMCA was performed using an automated Optimized Robot for Chemical Analysis (Beckman Coulter) programmed through the software SAMI (Beckman Coulter). Plates were then washed, fluorescein diacetate (FDA) added and the fluorescence generated was measured at 485/520 nm after 40-50 min incubation. A successful assay required a ratio of >10 between the signal in the control wells and the blank wells and a coefficient of variation of <30% in the control wells. The cytotoxic IC$_{50}$-value (inhibitory concentration 50%) was defined as the concentration giving a SI of 50%. The IC$_{50}$-values for drugs were determined from log concentration-effect (Survival Index %) curves in Graph Pad Prism (GraphPad software Inc.) using non-linear regression analysis. For the in vitro results, comparison of activity between two groups was made with two-sided t-test. Outliers were rejected using Dixon’s Q-test with a 95% confidence interval.

![FMCA assay description](image)

**Figure 6.** FMCA assay description

### Irradiation and clonogenic survival assay

In paper I, the radiosensitisation effect of the Hsp90 inhibitor 17-AAG in oesophageal cancer cells was determined by using a clonogenic survival assay (figure 7). Briefly, after 24 hour 17-AAG treatment, cells were irradiated with 2, 4, 6 or 8 Gy gamma photons. Following irradiation, single cell suspensions were prepared and counted, then plated into tissue culture dishes
at various cell densities for clonogenic cell survival analysis. Cells were incubated for 10-11 days, fixed with ethanol and stained with haematoxylin, and colonies of at least 50 cells were counted. A survival curve was constructed by plotting survival fraction against radiation dose. Radiosensitivity was quantified as area under curve (AUC) and the effect of 17-AAG on radiosensitivity was expressed as sensitiser enhancement ratio (SER), defined as $\frac{\text{AUC}_{\text{control}}}{\text{AUC}_{\text{treated}}}$. 

![Figure 7. Procedures for the clonogenic survival assay.](image)

Survival fraction = colonies counted / (cells seeded x $\text{PE}_{\text{control}}$).

**Telomerase activity assay**

In paper IV, telomerase activity from oesophageal cancer cell extracts was analysed by using TRAPeze kit (Chemicon) which is a PCR-based telomeric repeat amplification protocol (TRAP) assay described by Kim et al. (Kim *et al.*, 1994). Briefly, protein extracts were prepared according to manufacturer’s instruction and protein concentration was determined using the BCA protein assay reagent kit (Pierce chemical). The TRAP assay combines an elongation and amplification reaction; at the elongation reaction telomerase adds a number of telomeric repeats to the forward primer (TS) and in the second step, the extended products are amplified by adding the reverse primer generating a ladder of products with 6-base increments starting at 50 nucleotides. Reaction mixtures were then size-fractionated by electrophoresis in a 10% non-denaturing polyacrylamide gel and stained with SYBR Green 1 dye (Invitrogen). Gels were photographed using the Gel DocXR
photo system (Bio-Rad) and analysed with the Image Lab software (Bio-Rad). The relative telomerase activity was determined from the ratio of the entire telomerase ladder to the internal control.

Statistics

In paper I, survival was estimated using the Kaplan-Meier product limit method, with univariate analysis being performed using a log-rank test. Cox regression analysis was performed to investigate if certain continuous factors had a significant effect on survival. Throughout the work a 5% significance level was used.

In paper II, the survival functions were estimated with the Kaplan-Meier product limit estimator method and the median survival time estimated with linear interpolation of the survival function. The univariate statistical comparisons of the data were made using log-rank tests. Correlations to sensitivity for chemotherapeutic agents were analysed using Spearman rank order correlations. Kruskal-Wallis correlation test was used to determine statistical significance. Throughout this study, a 5% significance level was used.
Results and Discussion

Paper I: Hsp90 is expressed and represents a therapeutic target in human oesophageal cancer using the inhibitor 17-Allylamino-17-demethoxygeldanamycin

In this study, all investigated oesophageal tumours (81 patients) showed Hsp90 expression, but control oesophageal tissue expressed no or very low levels Hsp90. The observed data is in contrast to work by Faried et al., who demonstrated an expression of Hsp90 in only 50% of the tumours (123 cases), all cases being squamous cell carcinoma (Faried et al., 2004). Our study included both squamous cell carcinoma and adenocarcinoma, with squamous cancer dominating and all having a clear staining of Hsp90. In squamous cell carcinoma, a marked up-regulation of Hsp90 could be noted in dysplastic epithelium and invasive cancer compared to normal epithelium. In adenocarcinoma, Hsp90 was expressed in neoplastic epithelium and weakly also in normal non-neoplastic glands. Furthermore, squamous cell carcinomas expressed relatively higher Hsp90 levels compared with adenocarcinomas. Expression of Hsp90 in the oesophageal tumours was evaluated in relation to survival using both intensity and extent of staining. No significant correlation was found between survival and Hsp90 expression, neither for intensity (p=0.68) nor extent (p=0.38). Type of histology, squamous cell carcinoma or adenocarcinoma, did not make any difference (p=0.50 and p=0.93 for intensity, respectively). These results were in concordance with the data from Faried et al. (Faried et al., 2004) who found no correlation between prognosis and Hsp90 expression in oesophageal cancer patients. However, high Hsp90 expression was found to be associated with decreased survival in breast cancer patients (Pick et al., 2007). In our study, all tumours analysed displayed Hsp90 staining regardless of the disease outcome whereas normal tissue displayed no or weak staining, indicating that Hsp90 may represent a tumour selective therapeutic target in oesophageal cancer.

Cytoplasmic staining was found in 70% of the tumours whereas nuclear staining was detected in only 2.5% of the cases and the rest displaying both cytoplasmic and nuclear staining. These results are contradictory to previously reported data where nuclear localisation was found in 40% (10/25) of prostatic adenocarcinomas, but in none of the non-malignant specimens (Burkitt et al., 2007). A change of Hsp90 localisation in tumours may predict
a change in Hsp90 bound proteins which may influence the consequence of Hsp90 inhibition. The predominant cytoplasmic Hsp90-staining in our study may reflect the function of Hsp90 in stabilising signal transduction molecules in the cytoplasm. Furthermore, we found a strong expression of Hsp90 in all nine oesophageal carcinoma cell lines, which is consistent with previous reports in other cancer cells (Ferrarini et al, 1992; Liu et al, 1999; Pick et al, 2007).

In order to study the effect of Hsp90 inhibition on proliferation, oesophageal cancer cell lines Kyse70 and Kyse450 were treated with 17-AAG and a maximal reduction of proliferation was achieved with 500 nM 17-AAG for 48 h with 80% inhibition for Kyse70 and 84% for Kyse450. Treatment with 17-AAG significantly increased rates of apoptosis to 18% for Kyse70 and 13% for Kyse450 compared to control. This is consistent with other reports where 17-AAG treatment showed anti-proliferative activity, e.g. in cervical carcinoma, lymphoma and thyroid cancer, (Park et al, 2003; Schumacher et al, 2007; Schwock et al, 2008).

Using a clonogenic cell survival assay, Hsp90 inhibition by 17-AAG significantly sensitised cells for gamma photon radiation. Treatment with 17-AAG sensitised the radioresistant Kyse450 cells more than the radiosensitive Kyse70 cells with a sensitiser enhancement ratio (SER) of 2.1 and 1.5 for Kyse450 and Kyse70, respectively; the result is consistent with other reports that 17-AAG has a better sensitisation effect on radioresistant cell lines (Machida et al, 2005; Russell et al, 2003). A higher rate of apoptosis was noted when 17-AAG was combined with radiation treatment. The apoptosis rate for Kyse70 cells was approximately 12% and for Kyse450 cells approximately 18% at 6 Gy. A combination of 17-AAG and radiation treatment increased apoptosis rate to 32% for Kyse70 and 24% for Kyse450, significantly (p < 0.05) higher rates compared to radiation or 17-AAG treatment alone.

Further studies found that 17-AAG can potentially downregulate EGFR and also the activity of the downstream signalling proteins Akt and Erk1/2, correlating with decreased cell proliferation rate after 17-AAG treatment. The EGFR tyrosine kinase inhibitor gefitinib also effectively downregulated EGFR signalling in our experiments. However, gefitinib only slightly reduced the proliferation of Kyse70 and Kyse450 cells. Furthermore, gefitinib could not influence the radiosensitivity. It has been reported that Hsp90 has several client proteins. In the present study, we found that the IGF-1R and downstream signalling was also effectively inhibited by 17-AAG. Moreover, serum-induced tyrosine phosphorylation of several proteins of unknown identity was blocked by Hsp90 (data not shown). The radiosensitising effect of 17-AAG may be due to its ability to affect several cell signalling components, for example the EGFR and IGF-1R signalling networks in our study,
while gefitinib as a more selective compound was not able to radiosensitise the oesophageal cancer cells.

In conclusion, Hsp90 represents a novel therapeutic target in oesophageal cancer that can be targeted alone or in combination with radiotherapy. Hsp90 was selectively expressed in oesophageal cancer tissue. Inhibition of Hsp90 resulted in decreased proliferation and viability as well as radiosensitisation of oesophageal cancer cells by impeding several cellular components controlling cell proliferation and apoptosis.

Paper II: Expression of EGFR and LRIG proteins in oesophageal carcinoma with emphasis on patient survival and cellular chemosensitivity

Paper II investigated EGFR and LRIG1-3 expression using immunohistochemistry and their implications as clinical prognostic factors. A total of 80 patients were included in this study. The dominating histology was squamous cell carcinoma. EGFR and LRIG1-3 staining showed a predominant cytoplasmic localisation and correlation analysis demonstrated a significant correlation between EGFR and LRIG3 expression (p=0.006, R=0.37). This is in contrast with data from Guo et al. (Guo et al., 2007) who found a reverse correlation between LRIG1-3 and EGFR expression in the human pituitary adenoma cell line HP75. The other investigated parameters were not significantly correlated to each other or to clinical parameters. Survival analysis based on a total score of intensity and expression fraction showed a trend that low expression of EGFR (p=0.09) and high expression of LRIG2 (p=0.18) was associated with decreased survival, although not statistically significant. For LRIG1 (p=0.65) and LRIG3 (p=0.75) there was no significant correlation with survival. This is consistent with data on LRIG2 expression in cervical squamous cell carcinoma where high expression was associated with poor survival (Hedman et al., 2010). The fraction of EGFR expression has been reported to be associated with shorter survival in patients with oesophageal and oesophagogastric junction adenocarcinomas compared to those negative for EGFR (Kim et al., 2007). However, EGFR expression rates vary considerably, from 99% (Sarbia et al., 2007) to 29% (Kii et al., 2007) reported in oesophageal squamous cell carcinoma and both reports found no correlation between EGFR expression and survival. In our study, based on the score of staining intensity alone, high EGFR and LRIG2 staining intensity was statistically significantly associated with short survival (p = 0.025 and p = 0.03, respectively), while high expression fraction of LRIG1 was statistically significantly (p = 0.045) associated with long survival (figure 8).
We further investigated the importance of the expression levels of EGFR and LRIG proteins for sensitivity to conventional chemotherapeutic agents in nine oesophageal carcinoma cell lines. IC$_{50}$ values for seven standard cytotoxic drugs in nine oesophageal carcinoma cell lines were determined using the fluorometric microculture cytotoxicity assay (FMCA) and the RNA expression levels of EGFR and LRIG1–3 were evaluated by quantitative RT-PCR. Correlation analysis showed significant correlations between LRIG1 expression and sensitivity to cisplatin ($r = -0.74$), docetaxel ($r = -0.69$), and vinorelbine ($r = -0.82$). The correlation between LRIG1 levels and sensitivity to cisplatin is consistent with previously reported results where ectopic expression of LRIG1 resulted in increased sensitivity to cisplatin and temozolomide in malignant glioma cells (Stutz et al, 2008).
Figure 8. Kaplan-Meier curves demonstrating the survival differences for patients with different tumour EGFR staining intensity ($p = 0.025$) and LRIG2 staining intensity ($p = 0.03$), intensity graded 0–2, and also demonstrating the survival differences for LRIG1 staining ($p = 0.045$) when scored according to the proportion of positively stained tumour cells using a three-graded scale (0–2).
Paper III: Chemotherapeutic targeting of microtubules causes epidermal growth factor receptor dephosphorylation in oesophageal cancer cells

Paper III investigated the sensitivity of oesophageal cancer cells to microtubule targeting agents podophyllotoxin (PPT), vincristine and docetaxel by using a resazurin assay. Oesophageal cancer cell lines Kyse70 and Kyse140 responded to PPT, docetaxel and vincristine in a time- and dose-dependent fashion with a plateau-shaped curve which is consistent with tubulin inhibition and cell-cycle arrest effects. The microtubule targeting effect of PPT, docetaxel and vincristine was further proved by using immunofluorescence microtubule structure staining.

Since EGFR is commonly expressed in oesophageal carcinoma (Jankowski et al., 1993) and EGFR is highly expressed in all nine oesophageal carcinoma cell lines, we wanted to investigate potential effects of microtubule targeting on EGFR. The results showed that PPT and vincristine inhibited EGFR tyrosine phosphorylation after 24 h of treatment and depleted EGFR expression was pronounced after 48 h in both Kyse70 and Kyse140 cells. The general cytotoxic effect of PPT and vincristine after 48 h treatment might cause EGFR degradation and lead to EGFR downregulation, indicating that receptor phosphorylation and downregulation are two distinct events. In contrast, the microtubule stabilising agent docetaxel had a minor inhibitory effect on EGFR phosphorylation compared with PPT and vincristine. A substantial effect of docetaxel could only be observed after 48 h, indicating a possible indirect effect due to EGFR degradation.

To investigate cell signalling downstream of EGFR, we treated cells with drugs for 24 hours to avoid EGFR degradation. Phosphorylation of Akt was inhibited by docetaxel, vincristine and PPT in Kyse70 and Kyse140 cells. We noticed that the degradation of total Akt protein after drug treatment may explain the observed decrease in Akt phosphorylation. However, total Erk protein was not affected by drug treatment and phosphorylation of Erk was inhibited only by vincristine and PPT. IGF-1R was also found to be expressed in all nine oesophageal cancer cell lines in Western blot analysis. Microtubule targeting drug treatment had no effect on IGF-1R phosphorylation and expression, indicating receptor selectivity of microtubule targeting drugs.

The data suggested that EGFR dephosphorylation occurred after 24 hour drug treatment whereas substantial receptor downregulation occurred after 48 hour drug treatment, suggesting that these effects are independent from each other. To explore the mechanism of EGFR dephosphorylation, cells were treated for 24 hours with microtubule targeting drugs in the presence or
absence of the tyrosine phosphatase inhibitor sodium orthovanadate (Na$_3$VO$_4$). Western blot results showed that Na$_3$VO$_4$ abolished EGFR dephosphorylation. One possible explanation is that disruption of the microtubule network may release or activate a tyrosine phosphatase that can dephosphorylate EGFR. Sines et al reported that binding with tubulin could inhibit phosphatase activity of the phosphatases PTP$\varepsilon$ and PTP1b; PTP$\varepsilon$ activity was increased by the microtubule disrupting agent nocodazole (Sines et al, 2007). However, PTP1b or PTP$\varepsilon$ downregulation by siRNA did not influence the effect of the microtubule disrupting drugs on EGFR dephosphorylation in our study. In addition, we could not observe a significant increase of phosphatase activity of the phosphatases SHP-1, SHP-2, TCPTP, PTP1b and PTP$\varepsilon$ after disrupting microtubule structures in oesophageal cancer cells.

Paper IV: The effect of the telomerase antagonist imetelstat in oesophageal cancer cells

Paper IV studied the effects of imetelstat in oesophageal cancer cells as a potential therapeutic modality. Imetelstat is an oligomer antagonist that binds to the telomerase template RNA with a high affinity and inhibits the access of telomeres to telomerase, finally leading to shortening of the telomeres (Asai et al, 2003). Nine oesophageal cancer cell lines have different telomere lengths from 3.5 to 6.3 kbp. We selected Kyse520 (shortest telomeres) and Kyse410 (longest telomeres) for further studies. To evaluate the effect on telomerase activity, Kyse410 and Kyse520 were incubated with sense control and various concentrations of imetelstat for 72 hours. Telomerase activity assay using a PCR-based telomeric repeat amplification protocol (TRAP) showed that telomerase activity of Kyse410 and Kyse520 was significantly inhibited by 1$\mu$M imetelstat. An almost complete inhibition was observed at 5$\mu$M in Kyse520. No inhibition was observed after treatment with 10$\mu$M sense control.

Further, we examined the effects of imetelstat on long-term cellular growth, colony formation ability and cell cycle distribution. Kyse410 and Kyse520 cells were treated with 1$\mu$M imetelstat every three days and the cells were counted and re-seeded every week. Addition of imetelstat to Kyse410 (long telomeres) resulted in reduction of population doublings after 3-4 weeks, while Kyse520 (short telomeres) showed population doubling reduction after approximately 5-6 weeks. Thereafter, Kyse520 showed a more pronounced decrease of population doublings compared to Kyse410. After 30 weeks of treatment, Kyse410 had undergone 20 fewer population doublings compared to sense control, whereas Kyse520 had undergone 36 fewer population doublings. Thus, the decline of cell population doublings was more prominent in
Kyse520 with short telomeres and at the end part of the experiments there was an almost total growth abrogation in Kyse520. Cell cycle analysis showed that imetelstat treatment increased the percentage of cells in G1 phase in both Kyse410 and Kyse520. For Kyse410, imetelstat treatment for 14, 26, and 41 days resulted in approximately 20%, 13% and 5% more cells, respectively, in G1 phase compared to sense control treatment. For Kyse520, imetelstat treatment for 7, 13, 26 and 41 days resulted 6%, 20%, 5% and 15% more cells, respectively, in G1 phase compared to sense control treatment. These results are in agreement with other data published in lung and breast cancer where imetelstat treatment also resulted in an increase of G1 phase distribution (Dikmen et al., 2005; Gomez-Millan et al., 2007). Furthermore, pre-treatment with imetelstat (1 μM, two doses for one week) only formed around 40% colonies compared to sense control treatment for both Kyse410 and Kyse520, implying unknown effects of imetelstat that are independent of telomere dysfunction. Long-term pre-treatment with imetelstat for different time periods before the performance of clonal efficiency assay caused a successively impaired colony formation capacity in both cell lines.

Telomerase is important for DNA-damage repair and signalling (Masutomi et al., 2005). As shown in figure 9, both 53BP1 and γ-H2AX foci could be clearly detected and co-localised. We used 53BP1 foci staining for further analysis. Our study showed that 10 μM imetelstat treatment for three days resulted in significant increase of 53BP1 foci compared to sense treatment for both Kyse410 (p = 0.0078) and Kyse520 (p = 0.0002) cells, indicating a possible increase in DSBs. We then pre-treated Kyse410 and Kyse520 with imetelstat and sense control for three days and irradiated cells with 2 Gy. After 0.5 h, 1 h, 2 h and 24 h, we assessed the average number of 53BP1 foci per cell. Both Kyse410 and Kyse520 cells showed a peak of 53BP1 foci number per cell at 1 hour followed by a decrease, and the average number of 53BP1 foci per cell in cultures receiving the combined imetelstat/radiation treatment was higher compared to cultures treated with sense/radiation. For Kyse410, t-test showed a statistical significance at 0.5 h (p < 0.0001) and at time points 2 h and 24 h, the p values were close to statistical significance (0.055 and 0.066, respectively). For Kyse520, the average number of 53BP1 foci per cell in the imetelstat/radiation group was significantly higher than in the sense/radiation group at 0.5 h (p = 0.0015) and 24 hour (p = 0.0088). These data support the notion that telomerase is potentially involved in DSB repair in a telomere length-independent fashion. It has been reported that telomerase activity is up-regulated after exposure to radiation and telomerase protects cell from stress and DNA-damage (Masutomi et al., 2005; Ram et al., 2009). Furthermore, we analysed 53BP1 foci sizes and found that imetelstat/radiation treated cells had larger foci size compared to sense/radiation treated cells in both Kyse410 and Kyse520 at 2 h and 24 h (P < 0.05). Foci growth leads to amplification of DNA damage signals and cell cycle arrest when responding to low doses of ionizing radiation (Suzuki et al., 2011).
our study, the increased foci size in imetelstat-treated cells could be interpreted as increased damage severity which could lead to checkpoint activation.

Figure 9. Representative pictures show γ-H2AX (red), 53BP1 (green) foci staining and their co-localisation (yellow).
Conclusions

I  
Hsp90 was found to be selectively expressed in oesophageal cancer tissues compared to the corresponding normal tissues and abundantly expressed in nine oesophageal cancer cell lines. Inhibition of Hsp90 using the specific inhibitor 17-AAG led to a significant inhibition of cell proliferation and cell viability in oesophageal cancer cells. When combining 17-AAG and radiation treatment, apoptosis and clonogenic cell survival assays showed that inhibition of 17-AAG significantly sensitised oesophageal cancer cells to gamma photon radiation treatment. Further studies showed that inhibition of Hsp90 resulted in downregulation of EGFR and IGF-1R activation as well as downstream signalling, which correlated with the observed anti-proliferative, anti-survival and radiosensitisation effects induced by 17-AAG treatment. Thus, Hsp90 represents a potential therapeutic target in the treatment of oesophageal cancer, alone or in combination with radiotherapy.

II  
EGFR and LRIG proteins were expressed in oesophageal carcinoma. Based on a total score of intensity and expression fraction, a trend towards survival difference was found for LRIG2 and EGFR, but for LRIG1 and -3 there was no association with survival. Thus, EGFR and LRIG have limited prognostic implication in oesophageal carcinoma. Correlation analysis revealed a correlation between the expression of EGFR and LRIG3. Significant correlations between LRIG1 mRNA levels and sensitivity to cisplatin, docetaxel, and vinorelbine were found in nine oesophageal carcinoma cell lines. EGFR and LRIG proteins may be functionally involved and of predictive value in oesophageal carcinoma. However, larger materials are needed to fully elucidate the prognostic value of EGFR and LRIG proteins and their functional relationship in oesophageal carcinoma.

III  
Microtubule targeting by podophyllotoxin (PPT), vincristine and docetaxel led to inhibition of proliferation in oesophageal cancer cells. Furthermore, microtubule destabilising agents PPT and vincristine inhibited EGFR phosphorylation and, after prolonged exposure, EGFR stability. A protein tyrosine phosphatase inhibitor, sodium orthovanadate, largely abolished microtubule disruption-induced EGFR dephosphorylation. Thus, we propose that disruption of the microtubule network may lead to activation of a protein tyrosine phosphatase that dephosphorylates
EGFR, indicating an additional mechanism of action of microtubule targeting agents. However further efforts, such as siRNA-based screening experiments, are needed to elucidate the specific phosphatase involved.

IV Imetelstat, a telomerase antagonist, decreased proliferation and colony formation ability and inhibited telomerase activity in oesophageal cancer cells. Inhibition of telomerase activity by imetelstat led to an increase of 53BP1 foci indicating an induction of DSBs. Furthermore, the fraction and size of radiation-induced 53BP1 foci were increased after pre-treatment with imetelstat, supporting the notion that telomerase is involved in DNA repair processes. This study indicates that imetelstat is a potential therapeutic agent in the treatment of oesophageal cancer.
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