CHEMORES is an integrated project involving clinicians and scientists in eight European countries. The purpose of the project is to improve cancer treatment by obtaining increased knowledge on mechanisms of chemotherapy resistance. It is estimated that the two diseases that are studied, lung cancer and melanoma, caused over 350,000 deaths in Europe in 2002. An important contributing factor in cancer mortality is the fact that the most common types of cancer do not respond well to systemic chemotherapy in the advanced stages. Increased understanding of the underlying processes will contribute to the development of predictors of both therapy response and toxicity, and in the end more efficient and personalised therapy.

During the fourth year, the project has progressed essentially as planned and there have been extensive interactions between the participants. The collection of clinical samples, which has been the main challenge in the project, has largely been completed for the discovery studies and the project is gradually moving into the validation phase. In this period, CHEMORES has generated interesting findings in both clinical and pre-clinical studies and the progress in the different workpackages (WPs) are summarised below:

WP1 and WP3 – Clincal trials on melanoma patients

Work in WP3 overlaps with that of WP1 and WP2. In general, work during Period 4 has been partially focused on the collection of clinical materials for the discovery phase (a pilot phase of analysis to allow appropriate lab technology development has already been conducted); efforts have been made to obtain further samples for validation. Several tissue microarrays (TMAs) are currently being constructed for this purpose. This part of WP3 has been extended to allow for inclusion of more samples. Pilot samples of gene expression in formalin fixed tissues from patients with stage III melanoma participating in adjuvant trials of interferon therapy in high-risk melanoma showed that the technique works well. Gene expression profiling of tumors from stage IV patients treated with chemotherapy is under way.

WP2 and WP3– Clinical trials on lung cancer patients

The overall aim of WP2 is to generate biological material (blood and tumor specimens) and clinical data for patients with non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) who are treated with chemotherapy. A major and challenging goal is to provide paired tissue samples procured before and after chemotherapy for discovery of novel genes and pathways contributing to chemotherapy resistance, therapeutic escape and efficacy. In 2008, 110 patients were recruited to the CHEMORES project and archival frozen tumor and non-malignant lung tissue was obtained for 122 patients with complete clinical treatment and outcome data. These biological resources have been delivered to the genomics, proteomics and toxicity workpackages. In 2009 ~ 210 patients were recruited and a retrospective collection of frozen resected lung tumors was procured. In total 454 patients have now been recruited to the prospective study in 2010. Additional resources identified in 2010 include a 350 case squamous NSCLC TMA and a 120 case SCLC TMA.

WP4 – Genome analysis

During year 4, WP4 completed full exome sequencing of a panel of selected patients from the retrospective lung cancer material (NSCLC). The Bioinformatic group of IGR provided substantial advances in the effort of integrating and analyzing molecular data from the retrospective collection, in addition to the efforts of KI (WP6), and reported progress at the 2011 annual meeting in Manchester. WP4 completed molecular analyses of sequential biopsies collected for the temozolomide/sorafenib clinical trial in melanomas and developed the necessary tools for the investigation of formalin fixed paraffin embedded melanoma biopsies to address patients included in adjuvant IFN clinical trials.

WP4 also performed microarray experiments for the benefit of other WPs (Dr. Liaudet, Dr. Kroemer). WP4 received the frozen sequential biopsies for melanoma DTIC and TMZ clinical trials, as well as sequential biopsies from lung cancer before and after treatment and molecular profiling is ongoing. The objectives of WP4 in the upcoming period are to complete this molecular data set for all the sequential biopsies and to perform methylation microarrays for a panel of representative patients from the retrospective lung cancer cohort.

WP5 – Proteome analysis

We have performed lung cancer proteomics both on cell line material and clinical material to decipher proteome components relevant to lung cancer progression and therapy response. Novel methods for tumour proteome analysis and data analysis were developed, including studies of the phospho-proteome. Several altered pathways were detected and validation of prognostic markers in a clinical material is ongoing. A proteomics analysis of pre-treatment tumours from melanoma patients has also been completed. Several chemoresponse candidates were identified, including some that were previously identified by gene expression profiling. Validation will be performed during Period 5.

WP6 – Bioinformatics - Biostatistics

An algorithm to search for 'interesting' molecular subtypes, where 'interesting' is defined by the clinical objective such as prognosis or chemo-resistance, was developed during this period. The motivation comes from the fact that cancer is a heterogeneous disease, so different subgroups may use different molecular mechanisms for progression. We applied the algorithm to the retrospective lung cancer patient cohort and found an 8-gene signature that defined a molecular subtype with a strong prognostic significance. Furthermore, a network-based methodology to identify pathways that were likely activated for each patient is developed. The results conveyed the diversity of cancer development across patients. The activation score for each pathway is correlated with clinical phenotypes, such as histology, and with patient prognosis. Last but not least, a list of candidate markers for early diagnosis and therapy monitoring, based on genes that were differentially expressed between tumor and normal tissues is finalized.

WP7 – Animal tumor models

We have unveiled a synthetic lethal interaction between *K-Ras* oncogenes and *Cdk4* in a mouse tumor model that closely recapitulates human NSCLC. In this study, we also showed that conditional ablation of Cdk4 in lung adenocarcinomas induced rapid senescence leading to tumor regression of most of the tumors. Furthermore, we have initiated a long-term project to systematically examine, by genetic means, the contribution of the druggable Raf/Mek/Erk cascade of kinases in the K-*Ras* driven NSCLC model. For the melanoma studies, we have generated a mouse model for metastatic melanoma carrying a conditional knock-in for the $Braf^{V600E}$ mutation and a conditional deletion for the PTEN tumor suppressor. Current work is focused on treating melanoma bearing mice with the Plexxicon/Roche compound PLX4032/vemurafenib, and aimed to isolate resistant tumours for their characterization.

It was found that the responses of the *Adeno-Cre,* $Rb^{F/F}$, $p53^{F/F}$ mouse model for SCLC to the maximum tolerable dose of cisplatin differ from what is observed in the clinic. We continue to optimize this model. In parallel, the analyses of resistance to cisplatin, topotecan, docetaxel, doxorubicin and the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib in the *K14Cre,* $Brca1^{F/F}$, $p53^{F/F}$ and *K14Cre,* $Brca2^{F/F}$, $p53^{F/F}$ models were continued. We managed to induce docetaxel, doxorubicin or olaparib resistance in ABCB1;BRCA1;p53-deficient tumors and topotecan resistance in BRCA1;p53-deficient tumors which lack ABCG2. As a first result we found that 3/11 olaparib and 3/20 topotecan resistant tumors had lost 53BP1 expression, suggesting partial reversion of homology-directed DNA repair as another resistance mechanism.

WP8 – Lung cancer and melanoma stem cells

In the 4th period of CHEMORES, we collected data demonstrating that epithelial-mesenchymal transition (EMT) activation in a primary lung cancer cell line provides cells with mesenchymal traits as well as stem-like properties, as demonstrated by up-regulation of stemness genes, expansion of $CD133^+$ fraction and increased resistance to cisplatin treatment. Within the EMT-generated $CD133^+$ cells, we identified a subset of $CD133^+/ESA^-/CXCR4^{High}$ cells endowed with a mesenchymal and migratory phenotype. This cell fraction was enriched in patient lymph node metastases and experimental lung metastases, compared to parental lung tumours, suggesting that $CD133^+/ESA^-/CXCR4^{High}$ may represent the putative migrating cancer stem cells involved in the metastatic process. Thus, a distinct subset of $CD133^+$ migrating CSCs, possibly generated through the EMT process, could be responsible for metastasis formation. The ongoing molecular characterization of these populations will likely yield additional information about lung CSCs as well as additional targets for therapy.

Melanoma CSCs were selected *in vitro* as melanospheres, i.e. melanoma cells growing as non-adherent colonies. Primary and serially transplanted xenografts recapitulated the phenotypic features of the original melanoma of the patient. Melanospheres displayed a heterogeneous phenotype for the expression of stem cell markers. However, they displayed an enhanced expression of the embryonic markers Nanog and SOX3/4 as compared to adherent melanoma cell lines. Current efforts are aimed at dissecting the interaction between melanoma stem cells and the immune system. Melanospheres have been profiled for the expression of cell surface molecules involved in modulating immune response and for the production of chemokines and cytokines. We found that melanospheres secreted many factors implicated in melanoma development and progression. Moreover, initial experiments pointed out a role of some cytokines in modulating melanoma stem cell self-renewal.

WP9 – Novel therapeutic strategies

WP9 aims to develop novel therapeutic strategies for drug resistant cells via two major goals: reverse drug resistance associated with tumor acidification and development of novel compounds. A number of compounds that induce apoptosis of tumor cells have been identified by screening. The most interesting compound is an inhibitor of two cellular deubiquitinylating enzymes (DUBs) of the 19S proteasome: UCHL5 and USP14. These compounds induce apoptosis of drug resistant cells and of cells overexpressing Bcl-2. A number of structural analogues have been synthesized and tested. Robust effects have been observed in a SCID mouse xenograft model.

The DUBs induce cathepsin-D-dependent apoptosis by unclear mechanisms. A cathepsin-D interacting protein has been identified which is known to be involved in apoptotic processes. Experiments are ongoing to understand the importance of this partner for induction of apoptosis by compounds identified in WP9. Several compounds that induce apoptosis of cells grown in multocellular spheroids have also been identified by screening.

Proton pump inhibitors (PPIs) have been shown to induce cell death of human tumors. PPIs induced activation of caspase-3, -8, and -9, mitochondrial membrane depolarization and accumulation of reactive oxygen species. In addition, PPIs were found to induce autophagy in melanoma cells via inhibition of the mTOR pathway. PPIs are tested with the aim of increasing tumor pH and to improve therapy results. Our studies show that the PPI anti-tumor effect is consistent with a lowering of the pH gradient between the external and the internal microenvironments.

WP10 – Mechanisms involved in resistance to DNA damaging drugs

The transfer and expression of the *S. Pombe* gene product Atl1, which binds to O^6meG in DNA and strongly inhibits the action of MGMT *in vitro*, has been achieved in human tumor cells *in vitro*. This approach is being used to explore novel DNA damage response pathways conferring temozolomide (TMZ) resistance, and to explore an *in vitro* assay for the possible nucleotide excision repair of O^6-

alkylguanine. In addition, IGF1R inhibition has been shown to enhance the cytotoxicity of TMZ in human melanoma cells *in vitro* and we are now pursuing evaluation of this combination therapy *in vivo* in an animal model.

Deficient MMR is more common in tumors from non-responders than responders to DTIC/TMZ therapy, although it does not reach statistical significance (Fisher´s exact test; p=0.12). PARP1, however, is primarily a marker of aggressive behaviour, not an appropriate predictive marker of DTIC/TMZ response. The impact of MGMT promoter methylation as a predictive marker of chemotherapy response in melanoma is currently being investigated in a larger clinical material. Effects of targeted therapies alone and in combination with chemotherapies are studied in a panel of well characterized melanoma cell lines.

WP11 – Apoptosis regulation

The consortium has identified several microRNAs, mRNAs, proteins and processes that regulate the susceptibility of cancer cells to the induction of chemotherapy-induced cell death. The functional (genetic, biochemical and epistatic) analysis of these microRNAs, mRNAs, proteins and processes has been carried out and has been validated in preclinical studies. These results have allowed the design of strategies for sensitizing cancer cells to chemotherapy-induced cell death, by reducing the expression of resistance factors that protect cells against cell death inducers. Moreover, the clinical validation of these strategies is underway.

WP12 - Studies of markers of individual chemotherapy-induced toxicity

Blood samples for preparation of DNA for SNP analyses have now been collected from more than 500 NSCLC patients treated with platinum based chemotherapy in Paris, Manchester and Stockholm. Most patients have been treated with carboplatin in combination with either gemcitabine or vinorelbine. Data on treatment-related toxicity have been retrieved from clinical and laboratory records with focus on bone marrow toxicity (neutropenia and thrombocytopenia) and neurotoxicity.

We have previously made a list of candidate genes of importance for toxicity based on knowledge of metabolism and transport proteins for drugs used in lung cancer as well as a bioinformatics approach. Published data from gene expression arrays of cells or tissues exposed for drugs used in lung cancer have been collected and up- and down-regulated genes have been identified. 5,000 to 6,800 non-synonymous SNPs were identified and 3,100 to 6,400 insertions/deletions causing changes in about 100 proteins were found by using the bioinformatics approach. This investigation was validated by two strategies. Using the first strategy, 60 genes were compared based on a prior knowledge on effects of cytotoxic drugs. A strong correlation was found between ATP7A genes and neurotoxicity. In the second strategy, the allele distributions were compared in 100 genes selected by a meta-analysis of published results on genes differently expressed in microarrays of tissues after treatment with carboplatin and gemcitabine. A strong correlation was found between HLA-genes and neurotoxicity. Further analyses in another set of 8 + 8 patients (high and low toxicity) are in process and in-depth bioinformatic approaches will be used for whole exome comparisons.

PLAN FOR USING AND DISSEMINATING THE KNOWLEDGE

The knowledge produced within CHEMORES may be exploitable. Any innovations with such potential will be aided by the Technology Transfer Work Group which has put together a Technical Implementation Plan for the project.

With regard to dissemination to the public and the scientific community, the public website has been updated constantly during the period, to provide information about the project to interested parties, including the EC, user groups, other international research groups and the general public. In addition,

an internal newsletter has been distributed and a web-based knowledge management system is in place to facilitate the daily transfer of information between the participants. The dissemination of knowledge includes publications in scientific journals, presentations at scientific meetings and workshops.

CHEMORES Contractors

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Centro Nacional de Investigaciones Oncológicas, Spain
Erasmus University, Netherlands
Eurogentec, Belgium
European Organization for Research and Treatment of Cancer, Belgium
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