Hypermethylation as a mechanism for cisplatin resistance in germ cell tumors

Abstract

Theoretical framework: The molecular basis of cisplatin resistance in germ cell tumors (GCT) is poorly understood. As testicular germ cell tumors (TGCT) have rare somatic mutations, epigenetic alterations like hypermethylation of DNA have been proposed as possible mechanism for cisplatin resistance.

Hypotheses: The DNA methylation pattern of cisplatin-resistant tumors is different to cisplatin-sensitive tumors.

Methods: Tumor tissue samples from 84 patients with TGCT treated at the Department of Oncology at the Medical University of Graz will be retrospectively analysed with the EPIC array at the Department of Genetics at the Medical University of Graz and linked to clinical data. Methylation patterns of cisplatin-sensitive and cisplatin-resistant tumors will be compared to look for a difference in global hypermethylation and differentially methylated gene promoter and gene body regions.

Level of originality: A phase 1 study has shown promising results using the demethylating agent guadecitabine in cisplatin-resistant germ cell tumors. This could open a new therapeutic window for patients with cisplatin-resistant germ cell tumors. For future studies, which are beyond the scope of this project, we aim to additionally assess the epigenetic landscape *in plasma* using the Me-DIP-seq approach, which was recently published by a group of the Dana Farber Cancer Institute. We have already established a collaboration with the group of Christopher Sweeney.

Table of Content

1. Background and theoretical framework

1.1 Epidemiology and classification of testicular germ cell tumors

Testicular germ cell tumors (TGCT) are derived from germ cells and are classified as seminoma or non-seminoma. The latter may consist of one histology or be mixed non-seminoma.[1] The incidence of TGCT has risen considerably in Caucasian populations for more than 3 decades and is now the most common malignancy in men aged 15 to 40 years.[2] Yet precise molecular mechanism(s) responsible for the carcinogenesis of TGCT and their progression are still poorly understood.

1.2 Treatment of TGCT

The mainstay in the treatment of metastatic TGCT is cisplatin based combination chemotherapy. Outcome of patients with metastatic TGCT is dependent on tumor stage and tumor marker levels (prognosis groups). The five-year survival rates according to the International Germ Cell Cancer Collaborative Group risk classification range from 45-67% for the poor prognosis group to 96% for the good prognosis group. [3, 4] Cisplatin based chemotherapy represents the treatment for patients in the good prognosis group as well as for patients in the poor prognosis group, only varying in the number of cycles given (three or four cycles of BEP chemotherapy respectively). Several studies have investigated a more intense treatment approach for patients with poor prognosis, e.g. upfront high dose chemotherapy or switch to a more intensified treatment for patients with an unfavorable decline of the tumor markers alpha fetoprotein (AFP) and human chorionic gonadotropin (HCG) during conventional chemotherapy. Upfront high dose chemotherapy has failed to show a statistically significant improvement of overall survival in comparison to conventional BEP chemotherapy in a phase 3 trial. [5]

1.3 Complex genomic evolution of cured and resistant germ cell tumors

TGCT are characterized by frequent chromosomal anomalies such as gain of chromosome 12p and low rates of somatic mutations. [6, 7] The genes that are most commonly altered are *KIT, TP53 and KRAS/NRAS*. TP53 wildtype TGCTs are linked to chemosensitivity whereas somatic mutations in TP53 are associated with chemoresistance, although this represents a minority of resistant tumors. The emergence of chemotherapy resistance has been also linked to reciprocal loss of heterozygosity (RLOH), epigenetic alterations like hypermethylation and loss of pluripotency markers like NANOG and POU5F1. [1, 6] Studies have also revealed differential methylation and transcriptional status of more indolent or aggressive GCT histologic subtypes, which indicates additional mechanisms of reprogramming that may contribute to the initiation of RLOH and progression to the chemoresistance state. Taken together, these observations indicate that both genetic and epigenetic processes underly GCT development and progression, and that deep investigation of GCTs grounded in human tumor analysis can illuminate the causes and consequences of aggressive and lethal forms of this disease. By identifying the genetic basis underlying the origins of GCTs and discriminating features associated with treatment response, we can better understand why these cancers respond differently to treatments and develop methods to cure patients who would otherwise die of their disease.

1.4 Existing Results relevant to the Proposal

This proposal will be a cooperation between the Division of Oncology and the Institute of Human Genetics at the Medical University of Graz and the Dana Farber Cancer Institute in Boston, USA.
Together we are performing a comprehensive histone mark and methylation analysis integrated with RNA sequencing analysis with the goal to define the drivers of relapse and resistance to chemotherapy. Specific to the methylation work, the Medical University of Graz will be leading the EPIC methylation analysis with bisulphite sequencing on tumor tissue.
So far, the Medical University of Graz has used the EPIC array for methylation analysis in FFPE samples from eight patients with TGCT. We were able to show the global methylation pattern (Figure 1) as well as the pattern of specific gene promotor regions (Figure 2). For a comparison of cisplatin-resistant and cisplatin-sensitive tumors a larger sample size is needed.

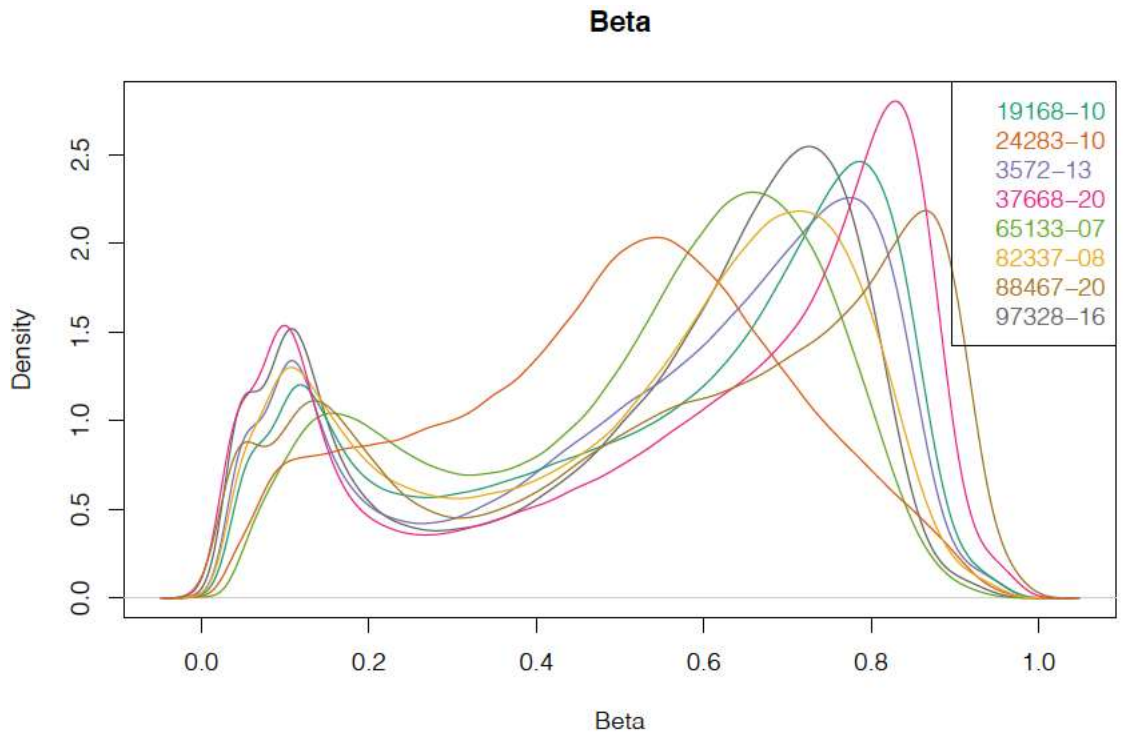

Figure 1. The global methylation pattern often follows a U-shaped distribution, i.e. peak of probes are unmethylated ~0 and another peak of probes methylated ~1. Notice that sample 24283-20 does not obey this distribution, most likely because of the poor quality of signal that dominated the sample.

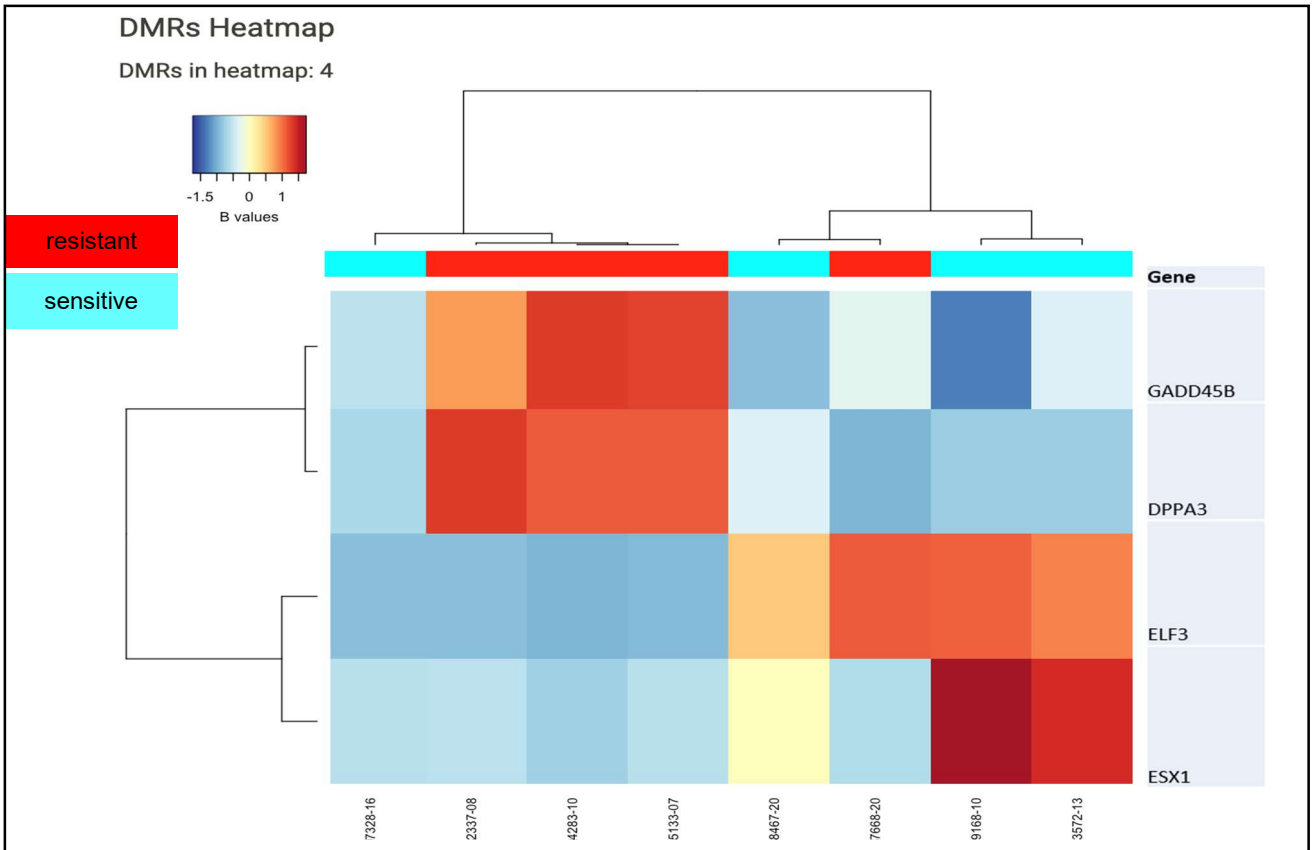

Figure 2. Heatmap of the 4 significant differentially methylated regions found in promoter regions when comparing chemosensitive and chemoresistant tumors (genes: GADD45B, DPPA3, ELF3, ESX1).

2. Rationale and Hypotheses of the Project

The main aim of the proposed project is to compare cisplatin-sensitive and cisplatin-resistant tumors and look for a different DNA methylation pattern in tumor tissue in these two groups. This project will be part of a cooperation between the Medical University of Graz and the Dana Farber Cancer Institute for comprehensive histone mark and methylation analysis integrated with RNA sequencing analysis with the goal to define the drivers of relapse and resistance to chemotherapy.

2.1 Hypotheses

Hypothesis I: Cisplatin-resistance in GCT is associated with global DNA hypermethylation

We will assess genome-scale DNA methylation in chemosensitve and chemoresistant samples. Tumor samples of patients who were cured with first line chemotherapy will serve as chemosensitive samples. Patients who relapsed after first line chemotherapy or died from their disease will serve as chemoresistant group. Analysis of Methylation-EPIC array data will be performed in R using minfi package from Bioconductor.

Hypothesis II: Chemoresistant and chemosensitive samples can be distinguished by differentially methylated gene promoter and gene body regions.

As studies from cell lines have shown that tumour suppressor gene repression in cisplatin-resistant cells might be controlled by location-specific CpG hypermethylation, we will look for differentially methylated regions between chemoresistant and chemosensitive samples.

3. Significance and Originality of the Project

Genomic analysis of TGCT patients will lead to better understanding of the biology of these tumors. The cooperation between the Medical University of Graz and the Dana Farber Cancer Institute will share specimens to conduct studies of the methylation and histone mark epigenetic assays to predict relapse and resistance to chemotherapy.

Firstly, these methylation analyses can have a prognostic and predictive value for patients with GCT.

Secondly, for future studies, which are beyond the scope of this project, we aim to additionally assess the epigenetic landscape in plasma using the Me-DIP-seq approach, which was recently published by a group of the Dana Farber Cancer Institute.

Thirdly, our work can lead to further therapeutic investigations with hypomethylating agents in patients with chemoresistant GCT. A phase one study has shown promising results with the hypomethylating agent guadecitabine [8].

4. Methods

This is a retrospective analysis of collected tissue samples from 84 patients with GCT who have been treated at the Medical University of Graz. Slides from these 84 patients have also been shipped to the Dana Farber Cancer Institute for comprehensive histone mark analysis integrated with RNA sequencing analysis with the goal to define the drivers of relapse from localized disease and resistance to chemotherapy.
For this proposed part of the project methylation analysis will be performed using the EPIC array at the Medical University of Graz.

4.1 DNA Extraction

DNA will be purified using the Qiagen DNeasy Kit at the Department of Pathology at the Medical University of Graz.

4.2 DNA analysis

To identify somatic copy number alterations (SCNA) and to estimate the tumor content we will employ sWGS and analyze the data with the ichorCNA algorithm. Only samples that yield informative results will be used for further analysis (quality check).

4.3 Methylation analysis

The Infinium EPIC beadchip array will be used to assess the methylation level of CpG sites. It targets 863,904 CpG sites.

4.4 Statistical considerations

4.4.1 Sample size

The sample size calculation was performed using a two-sided *t*-test to detect methylation differences between two groups and according to recommendations for Illumina Epic Array studies.[9] A sample size of 84 patients (21 patients with chemo-resistant and 63 patients with chemo-sensitive tumors) achieves 87% power to detect a mean difference of 5% in methylation patterns at single CpG sites with a standard deviation of ≤ 0.02, if a t-test is used with the recommended genome-wide significance level of 9.42×10^{-8}.

4.4.2 Data analysis

Analysis of Methylation-EPIC array data will be performed in R using minfi package from Bioconductor. Data analysis will be performed in-house by the bioinformaticians at the Institute of Human Genetics. Quantitative genetic changes will be analyzed by nonparametric repeated measures analysis. Associations between genetic changes and clinical variables

will be assessed by nonparametric tests. The influence of genetic changes on survival time accounting for potential confounding factors will be analyzed by Cox regression

5. Cooperations

5.1 Institute of Human Genetics at the Medical University of Graz

After DNA extraction, SCNA analysis will be performed in all samples for quality purposes. Methylation analysis will then be performed in samples with enough tumor content using the EPIC array. All basic laboratory equipment, needed for diagnostic and research applications is available. Hence, the Institute of Human Genetics is completely equipped for all proposed experiments and therefore no funding for equipment is requested.

5.2 Dana Farber Cancer Institute

The proposed project is part of a collaboration with the Dana Farber Cancer Institute with the goal to define the drivers of relapse and resistance to chemotherapy. Specific to the methylation work, the Medical University in Graz will be leading the EPIC methylation analysis with bisulphite sequencing.

6. Time plan

1st Year	2nd Year
DNA extraction of already collected samples, SCNA, Methylation analysis	
	Data analysis, manuscript writing, publication

7. Qualifications of the contributing scientists

7.1 Principal Investigator

The grant applicant and project leader, Priv. Doz. Dr. Angelika Terbuch, has achieved the position of habilitation in Internal medicine at the Medical University of Graz. Her main research as an oncologist so far has focused in the area of genitourinary cancers with a special interest in testicular cancer. Her world-wide collaborations have led to successful publications in high-rank journals and she has been part of the consortium for the update of the International Germ Cell Cancer Collaborative Group risk classification. To continue her research and achieve the

position of an associate professor at the Medical University of Graz funding of the project as described would be essential for her.

7.2 Co-Investigator

The Co-Investigator, Assoc.-Prof. PD. Mag. Dr. Ellen Heitzer, has achieved the position of an associate professor at the Institute of Human Genetics at the Medical University of Graz. She is one of the leading scientists in Austria dealing with liquid biopsies and has developed a set of techniques for the analysis of ctDNA. Prof. Heitzer was granted a Christian Doppler Laboratory for liquid biopsies in the context of early cancer detection.

7.3 Co-Investigator

The Co-investigator, Prof. Christopher Sweeney is a medical oncologist with a major focus on prostate cancer and testicular cancer. He is a physician who conducts translational research with a track record of making significant contributions to field with research emanating from the laboratory and the clinic. In 2004 he was given the responsibility and honor of writing and being the principal investigator of the Eastern Co-operative Oncology Group trial, *E3805: CHAARTED trial – Chemohormonal therapy versus androgen ablation randomized trial in extensive disease prostate cancer.* He was granted a NIH grant to investigate molecular origins and evolution to chemoresistance in germ cell tumors.

8. Ethical aspects

Tissue samples have been stored at the biobank of the Medical University of Graz. The Ethics Committee of the Medical University of Graz has approved the study (32-527 ex 19/20).

9. References

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10. Costs

Consumables				
Application	**Samples analyzed**	**Item**	**Price/ sample**	**Total**
FFPE	84	Bisulfite conversion and Infinium Methylation EPIC array analysis	300.00	€ 25.200.00

If this project will be funded by the OEGHO with 15.000 Euros, the rest of the costs will be carried by the Dana Farber Cancer Institute.

11. Curriculum of the Principal Investigator

Present academic position and business address

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Consultant position at the Department of Internal Medicine,
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Education

2020	Habilitation in Internal Medicine
2019 until up to date	Consultant at the Department of Internal Medicine, Division of Oncology, Medical University of Graz, Austria
2017 - 2018	Research fellowship at the Drug Development Unit, Royal Marsden Hospital, Sutton, United Kingdom
2013 - 2017	Residency in Internal Medicine, Division of Oncology, Medical University of Graz, Austria
2011 - 2013	Residency in Internal Medicine, Department of Hematology and Oncology, Paracelsus Medical University of Salzburg, Austria
2007 – 2011	Training as General practitioner, Klinikum Wels-Grieskirchen, Austria Cheltenham General Hospital, United Kingdom
1999 - 2006	Medical Degree; Medical University of Graz, Austria,

Member of the following societies:

Austrian Society of Hematology and Oncology (ÖGHO), American Society for Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO)

Research grants:

Hans und Blanca Moser Stiftung: MiR-371a-3p Serumlevels are increased in recurrence of testicular cancer

ESMO Research Fellowship 2017: Liquid biopsy by apheresis

Publications of the last 5 years (10 most important in peer reviewed journals):

2021:

Gillessen S, Sauvé N, Collette L, Daugaard G, de Wit R, Albany C, Tryakin A, Fizazi K, Stahl O, Gietema JA, De Giorgi U, Cafferty FH, Hansen AR, Tandstad T, Huddart RA, Necchi A, Sweeney CJ, Garcia-Del-Muro X, Heng DYC, Lorch A, Chovanec M, Winquist E, Grimison P, Feldman DR, **Terbuch A**, Hentrich M, Bokemeyer C, Negaard H, Fankhauser C, Shamash J, Vaughn DJ, Sternberg CN, Heidenreich A, Beyer J; International Germ Cell Cancer Classification Update Consortium.
Predicting Outcomes in Men With Metastatic Nonseminomatous Germ Cell Tumors (NSGCT): Results From the IGCCCG Update Consortium. J Clin Oncol. 2021 Apr
DOI: 10.1200/JCO.20.03296.

Beyer J, Collette L, Sauvé N, Daugaard G, Feldman DR, Tandstad T, Tryakin A, Stahl O, Gonzalez-Billalabeitia E, De Giorgi U, Culine S, de Wit R, Hansen AR, Bebek M, **Terbuch A**, Albany C, Hentrich M, Gietema JA, Negaard H, Huddart RA, Lorch A, Cafferty FH, Heng DYC, Sweeney CJ, Winquist E, Chovanec M, Fankhauser C, Stark D, Grimison P, Necchi A, Tran B, Heidenreich A, Shamash J, Sternberg CN, Vaughn DJ, Duran I, Bokemeyer C, Patrikidou A, Cathomas R, Assele S, Gillessen S; International Germ Cell Cancer Classification Update Consortium.
Survival and New Prognosticators in Metastatic Seminoma: Results From the IGCCCG-Update Consortium.
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Dandachi, N; Posch, F; Graf, R; Suppan, C; Klocker, EV; Müller, HD; Lindenmann, J; **Terbuch, A**; Heitzer, E; Balic, M**.**
Longitudinal tumor fraction trajectories predict risk of progression in metastatic HR^+ breast cancer patients undergoing CDK4/6 treatment. Mol Oncol. 2020; 4(1)
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2020:

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Yap, TA; Tan, DS; **Terbuch, A**; Caldwell, R; Guo, C; Goh, BC; Heong, V; Haris, NRM; Bashir, S; Drew, Y; Hong, DS; Meric-Bernstam, F; Wilkinson, G; Hreiki, J; Wengner, AM; Bladt, F; Schlicker, A; Ludwig, M; Zhou, Y; Liu, L; Bordia, S; Plummer, R; Lagkadinou, E; de Bono, JS.
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Lu, C; **Terbuch, A**; Dolling, D; Yu, J; Wang, H; Chen, Y; Fountain, J; Bertan, C; Sharp, A; Carreira, S; Isaacs, WB; Antonarakis, ES; De Bono, JS; Luo, J.
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2019:
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DOI: 10.3390/ijms19103130

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2017:
Bezan, A; Posch, F; Ploner, F; Bauernhofer, T; Pichler, M; Szkandera, J; Hutterer, GC; Pummer, K; Gary, T; Samonigg, H; Beyer, J; Winder, T; Hermanns, T; Fankhauser, CD; Gerger, A; Stotz, M.
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12. Letter of Collaboration

DANA-FARBER CANCER INSTITUTE | BRIGHAM AND WOMEN'S HOSPITAL

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October 6, 2021

OeGHO
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Dear Review Committee Members.

It is with great enthusiasm that I write this letter of collaboration for Dr. Angelika Terbuch's grant application entitled "Hypermethylation as a mechanism for cisplatin resistance in germ cell tumors", submitted to the OeGHO.

I am writing this letter of collaboration in my capacity as a Medical Oncologist at Dana-Farber Cancer Institute and as a Professor at Harvard Medical School and have been collaborating with Dr. Terbuch on the biological interrogation of germ cell tumor specimens. We meet one per month via zoom and exchange many emails in between meetings and have formed a close collaboration. Dr. Terbuch is part of our germ cell tumor research team as we are performing a comprehensive histone mark and methylation analysis integrated with RNA sequencing analysis with the goal to define the drivers of relapse from localized disease and resistance to chemotherapy. Specific to the methylation work, Dr. Terbuch's team is leading the EPIC methylation analysis with bisulphite sequencing while the DFCI team is developing the Me-DIP-seq approach. Our team effort will be the only comprehensive methylation analysis to define the benefits and ability of both techniques in FFPE and frozen tissue as well as circulating tumor cells. If the Me-DIP.seq approach performs as robust as the EPIC methylation assay we will further analyse liquid biopsy samples with the Me-DIP-seq approach as recently published by our centre in renal cell carcinoma patients. Our team effort also will share specimens to conduct well powered training and validation correlative studies to determine the prognostic and predictive capabilities of the methylation epigenetic assays to predict relapse and resistance to chemotherapy. The support of the OeGHO for the work conducted in Austria will be a major source of support to enable this very important project.

Sincerely,