# Translational Biology and MicroRNAs

## GCT-44 An overview of circulating microRNAs for the management of germ cell tumours

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**Background:** The current biomarkers AFP/HCG have limited sensitivity/specificity for diagnosing malignant germ-cell-tumours (GCTs). MicroRNAs are non-protein-coding RNAs that regulate gene expression. We previously showed that microRNAs from the miR-371–373 and miR-302/367 clusters are universally overexpressed in all malignant GCT tissues, regardless of patient age, tumour site or histological subtype. These microRNA clusters are not co-ordinately over-expressed in any other cancer type or disease state. These microRNA characteristics lend themselves to being promising candidate biomarkers.
**Material and methods:** The Cambridge team developed a highly sensitive pre-amplified qRT-PCR technique for the robust detection of microRNAs from the miR-371-373 and miR-302/367 clusters in circulating biospecimens from patients with malignant GCTs, now adopted by multiple other groups. The pipeline includes quality control checks and the use of an exogenous spike-in control and the endogenous microRNA miR-30b-5p for normalisation purposes, prior to data analysis.
**Results:** Results from our and other groups show that a four-serum miRNA panel (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) shows high sensitivity/specificity for diagnosing malignant GCTs. Of these, miR-371a-3p individually shows the most utility as it is most dynamic and most accurately reflects disease activity. These microRNA levels are useful for disease-monitoring and early detection of relapse. They should improve future clinical management of patients with malignant GCTs, in particular reducing CT scans in follow-up and identifying patients with apparent clinical stage I (CSI) seminoma who have persistently elevated serum microRNA levels post-orchidectomy (suggestive of micrometastatic disease), who may benefit from adjuvant chemotherapy to prevent subsequent recurrence.

## GCT-45 Insights into the mechanisms of expression and (specific) secretion of microRNA (-371a-3p) in malignant paediatric and adult germ cell tumours: towards a clinical applicable protocol

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**Background:** An intriguing characteristic of all malignant germ-cell-tumours (GCT) is expression of microRNA cluster miR-371-3 on chromosome 19, normally only found during embryonic development. No explanatory mechanism has been identified yet, although the promotor shows consistent demethylation. The specific member of this cluster, miR-371a-3p, has been proven to be highly informative as a liquid biopsy-based biomarker (serum/plasma and cerebrospinal fluid) at diagnosis and during follow-up. However, relevant information is still lacking.
**Methods:** Haemolysis effects on serum/plasma results in >700 samples, obtained by magnetic bead-based miR-purification protocol, was studied. Detailed evaluation was done to elucidate mechanism(s) of miR(-371a-3p) expression/secretion. Therefore (GCT) cell lines (n = 5) with matched conditioned media, mouse xenografts and matched plasma, as well as primary GCT and matched serum were investigated using a high-throughput miR-profiling approach. In addition, cell lines were cultured under various *in vitro* conditions, to investigate the mechanism of miR secretion.
**Results:** Haemolysis only impacts in case of high severity. A significant difference between serum and plasma was identified on the normalization target miR-30b-5p (but not ath-miR-159 used for calibration). Therefore, serum and plasma samples should not be used in same evaluation. A highly selective, and stable, secretion pattern was identified compared with cellular expression. Secretion was highly robust and extremely fast (within minutes), temperature/proliferation-independent, and exosome-mediated. While all miR-371a-3p molecules were exosome-packaged, this was only partial for miR-30b-5p. No improvement of assay-sensitivity was obtained using exosome-purification instead of miR-bead-based isolation. These data are relevant in development of final standardized miR-371a-3p detection protocols for clinical implementation.

## GCT-46 Serum microRNA-371a-3p levels predict viable germ cell tumour in chemotherapy-naïve patients undergoing retroperitoneal lymph node dissection

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**Background:** Serum microRNAs (miRNAs) are emerging as candidate biomarkers for diagnosing and monitoring germ cell tumours (GCTs). However, the ability of miRNA to inform treatment in low-stage chemotherapy-naïve patients has not been explored. We evaluated the performance of serum miRNA to predict viable GCT in chemotherapy-naïve patients undergoing primary retroperitoneal lymph node dissection (RPLND).
**Methods:** We prospectively collected presurgical serum samples from consecutive GCT patients undergoing primary RPLND at our institution from 2016–2019. Serum miRNAs (-367-3p/-371a-3p/-372-3p/-373-3p/-375) were isolated and quantified. RPLND histopathology was categorized as benign, viable GCT, or teratoma. miRNA levels were compared among groups. Receiver operating characteristic (ROC) curves were used to assess the discriminative ability of each miRNA signature to predict viable GCT.
**Preliminary Results:** 24 patients with stage I-II GCT underwent primary RPLND, revealing viable GCT in 11 (46%), teratoma in 3 (13%), and benign pathology in 10 (42%) patients. miR-371a-3p was the most discriminatory serum miRNA for viable GCT, exhibiting ~13,000-fold increase in expression over benign pathology. On ROC analysis, miR-371a-3p had AUC = 0.965, with sensitivity and specificity of 100% and 92%, respectively. AUCs for other serum miRNAs in predicting viable GCT were 0.874 (miR-367-3p), 0.846 (miR-372-3p), and 0.720

(miR-373-3p). These miRNAs were not predictive of pure teratoma. Serum miRNAs, particularly miR-371a-3p, can accurately differentiate small-volume viable GCT from benign processes or teratoma in patients undergoing primary RPLND, even with normal serum tumour markers. If validated in larger cohorts, these data suggest a basis to implement precision medicine strategies in treating patients with early-stage GCT.

## GCT-47 Targeting oncogenic microRNAs in malignant germ cell tumours with locked nucleic acid-based inhibitors

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**Background:** MicroRNAs (miRNAs) are short, non-protein-coding RNAs that regulate gene expression, primarily through mRNA degradation. We have shown that all malignant germ-cell-tumours (mGCTs) overexpress two miRNA clusters, miR-371~373 and miR-302/367, resulting in downregulation of functionally significant mRNAs/pathways. Here, we inhibited these overexpressed miRNAs in mGCT cells *in vitro* to determine their functional significance and explore their potential role as therapeutic targets.
**Methods:** qRT-PCR confirmed that miR-371~373 and miR-302/367 levels in mGCT cell lines [seminoma (SEM), yolk sac tumour (YST) and embryonal carcinoma (EC)] corresponded to those in tissue samples of the relevant tumour subtype. MiRNA inhibitors composed primarily of locked nucleic acids (LNAs) were designed to target key mature miRNAs. Gene expression profiling of cells treated with inhibitor or control was performed using mRNA microarray. *Sylamer* analysis was used to identify direct mRNA targets of these miRNAs, and key pathways analysed using KEGG and Reactome for functional validation.
**Results:** Targeting mature miRNAs of the miR-302 family (miR-302a-d) using LNA-based inhibitors resulted in reproducible growth inhibition in SEM and YST cells, although EC cells were more resistant to treatment. In the mGCT cells with reduced growth, *Sylamer* revealed enrichment of upregulated mRNAs that contained the seed-complementary-region for miR-302a-d in their 3' untranslated regions. Pathway analysis of these upregulated mRNA targets primarily demonstrated involvement in cell cycle regulation. This was functionally validated through cell cycle analysis using flow cytometry. LNA-based miR-302 family inhibition results in cell cycle arrest in two main subtypes of mGCT and may represent a new therapeutic approach.

## GCT-48 Analysis of circulating cell-free DNA identifies several gene mutations associated with germ cell tumours

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**Background:** Circulating cell-free DNA (cfDNA) has been described in various malignancies as a diagnostic and prognostic biomarker. We analyzed the significance of cell-free DNA in children with germ cell tumours (GCTs).
**Methods:** Cell-free DNA was isolated from the serum of 20 patients with GCTs, including 3 mature and 2 immature teratomas, and of 5 healthy individuals and then analyzed by next generation sequencing (NGS), using a custom cancer hot-spot panel covering hot-spot regions of 50 genes.
**Results:** In a total of 25 cfDNA samples analyzed, 8 had a pathogenic mutation in target genes, such as *TP53*, *IDH1*, *NOTCH1*, *PIK3CA*, *PTEN*, *SMAD4*, and *SMARCB1*. Seven tumours with pathogenic mutations in genes were advanced stages of mixed germ cell tumours, embryonal carcinoma, choriocarcinoma or yolk-sac tumour. One immature teratoma case showed a SMARCB1 mutation, which contained nephroblastoma component in this tissue. No hot-spot mutations were detected in the healthy individuals. Circulating cfDNA analysis using targeted NGS provided multi-gene detection that can be useful as a noninvasive and senstive tool for mutation profiling in GCT patients. This technology might provide both diagnostic and prognostic information in GCT. We should select target genes for practical and suitable cfDNA analysis in clinical settings for GCTs.

## GCT-49 Mechanism and functional significance of downregulated microRNA expression in malignant germ cell tumours

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**Background:** Chemotherapy for malignant germ-cell-tumours (mGCTs) results in substantial long-term side-effects. Accordingly, identifying novel therapeutic targets is a priority. MicroRNAs are short, non-protein-coding RNAs that regulate gene expression. We previously showed that miR-99a-5p/miR-100-5p (which are functionally synonymous) and miR-125b-5p are universally under-expressed in mGCTs. Here, we investigate the causes and consequences of such under-expression.
**Methods:** qRT-PCR suggested that miR-99a-5p/miR-100-5p and miR-125b-5p levels were co-regulated in mGCT cell lines [seminoma (SEM), yolk-sac-tumour (YST), embryonal carcinoma (EC)], as well as corresponding tumour tissue subtypes. Cells were treated with the DNA-demethylation agent 5′-azacytidine and pyrosequencing done to elucidate upstream causes of microRNA under-expression. Mimics for miR-100-5p and miR-125b-5p were used in combination to treat mGCT cells *in vitro*, compared with control-treated cells. Gene expression profiling was performed using mRNA microarrays. The bioinformatics algorithm *Sylamer* was used to identify direct mRNA targets of the miRNAs and the cellular pathways affected.
**Results:** 5′-azacytidine treatment caused up-regulation of all three miRNAs (miR-99a-5p/miR-100-5p/miR-125b-5p). Pyrosequencing revealed DNA-hypermethylation of the miR-99a-5p/-100-5p and miR-125b-5p loci in mGCT cell lines. Combined miR-100-5p/miR-125b-5p mimic treatment resulted in reproducible growth inhibition in SEM and YST cells, although EC cells were more resistant. In mGCT cells with reduced growth, *Sylamer* revealed enrichment of downregulated mRNAs that contained the seed complementary region for miR-100-5p/miR-125b-5p in their 3′ untranslated regions. These downregulated mRNA targets were primarily involved in pro-proliferative pathways. miR-99a-5p/miR-100-5p and miR-125b-5p are silenced by DNA-hypermethylation and are potentially important in mGCT progression. Replenishment of these miRNAs may offer a novel therapeutic approach.