PROJECT SWENOTECA MIR-STUDY

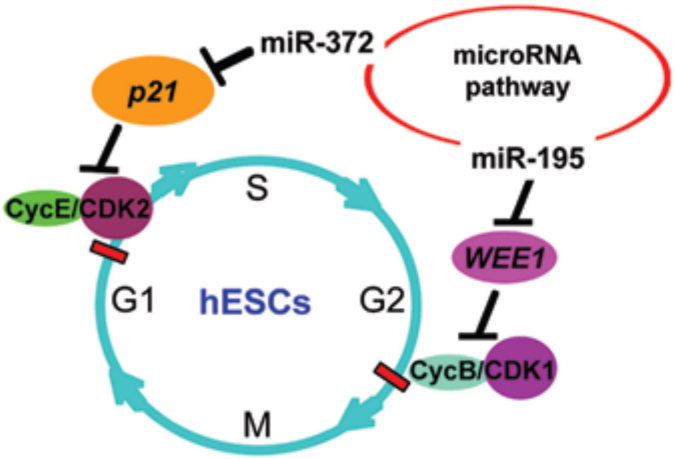
MicroRNAs as markers for disease activity and a tool to monitor the effect of chemotherapy and early detection of recurrence in patients with testicular germ cell tumours

Treatment of testicular cancer is a success story as we currently can cure about 97 % of all non-seminoma patients and 99 % of all seminoma patients [1-3]. The treatment is however associated with severe and potential lethal toxicity [4-8]. We and others have therefore continuously focused the research on less intensive therapy for subgroups of patients like stage I non-seminoma patients, where we select patients based on invasion into vascular structures [2], and now limit the treatment to one course of the standard cisplatin based BEP regimen [9]. For stage I seminomas we have stepwise limited the radiation treatment fields and radiation doses, and currently give only one course of carboplatin or observation alone (surveillance) based on presence of morphological risk factors observed in the primary tumour [3]. We have also successfully managed to cure the majority (77 %) of patients with metastases from non-seminomas by 3 -4 courses of the BEP regimen, reserving intensification with ifosfamid for slowly responding lesions (18%) and only apply intense high dose therapy for the 5 % with treatment resistant tumours [1,10]. This is in contrast to the most used approach internationally where the patients are allocated to various intensified regimens based on their clinical presentation. The key factors in the response adopted therapy for metastatic disease, is close monitoring of the treatment effect by serial measurements of the specific tumour markers α-fetoprotein (AFP) and human choriogonadotropin β (hCGβ) or roentgenological registration of tumour shrinkage [1]. These serum markers have now been included in the international UICC classification TNM classification system [11]. There is ,however, problems with the monitoring of serum markers as many patients continues to have moderately elevated markers which can lead to unnecessary chemotherapy intensification or too many chemotherapy courses, leading to unwanted side effects. There is evidence that the marker hCGβ has a biphasic elimination curve and that serum measurements reflect slow plasma elimination rather than continuous production in vital residual tumour cells [12] . For the largest subgroup, seminomas, we currently lack reliable serum markers, except lactate dehydrogenase (LDH) which may be elevated in bulky tumours. Thus there is a need for better markers to follow the disease activity in testicular germ cell tumours.

There is now extensive evidence for presence of stem cell populations in in many cancer types [13,14]. Such cells promote progression and spread of the cancer, but has also been identified as more chemotherapy resistant than the other tumour cells [15,16]. It is now known that there is expression of several specific proteins which keep the pluripotent capacity in embryonic tissues (i.e. OCT3/4, NANOG, SOX2, Klf4 etc) [17]. These factors seems also able to reprogram somatic cells into a pluripotent state [18]. Testicular cancer develops from developing germ cells, and the precursor lesion *cancer in situ* as well as seminomas and embryonal carcinoma type of non-seminomas express the stem cell markers NANOG,

OCT3/4, SOX2, SOX 17 among other markers [19]. These markers seem suitable to characterize the different morphological subtypes of testicular tumours, but their clinical role as prognostic or predictive factors is currently unknown. Therefore we do not know the drivers behind the development of testicular tumours. Exome sequensing of malignant testicular cancers from patients with bilateral tumours indicate that the different tumours does not share similar gene drivers [20].

Different microRNAs (miRs) regulate human embryonic stem cell division [21]. Experimental studies have revealed a double negative loop between the pluripotency proteins Oct4, SOX2 and KLF4 versus miR-145 such that miR-145 is low in embryonal stem cells and high in differentiated tissues [22]. Also the miR let-7 inhibits the reprogramming by the pluripotency factors by stimulating differentiating genes [23]. Other studies show that the miR-372 inhibit p21 and miR-195 inhibit WEE1 which also inhibit the cell cycle progression at G1-S and G2M transition, respectively (See figure) [24].



From Qi et al. [24]

The pluripotensy factors OCT3/4, SOX2 and NANOG are also under control of other miRs, where particularly the miR-371-373 cluster and miR302a-d and 367 cluster seems to be important [19,25]. MiR-373 is regulated by transforming growth factor beta (TGFβ), while miR-373 on the other hand also increases the level of TGFβ by inhibiting the TGFβ inhibitor Lefty1/2 [26,27]. Also miR-302-367 stimulate pluripotency characteristcs by acting on inhibitors of TGFβ and several other cellular pathways [26,28]. Mir-302a is also involved in response of testicular germ cell tumours to cisplatinby lowering the threshold for apoptosis [29].

Preliminary clinical studies

The first clinical report on expression of miR 3371-373 in panels of human testicular germ cell tumours came from Looijengas group [30,31]. Then both miR 302 and miR 372-373 was confirmed in tissues from pediatric germ cell tumours and some adult testicular cancer tissues [32,33]. These authors could also show that miR 302 and miR 371-373 members were elevated in serum from a four year old boy with a yolk sac tumour, which returned to normal levels during follow up [34] . In a pilot study with 20 stage I testicular cancer patients and 4

with metastatic disease, the miR 371-373 were elevated before diagnosis and declined rapidly after orchiectomy, and normalized more slowly during chemotherapy for the metastases [35-37]. In a separate study it was documented increased consentration of miR 302 and miR 371-3 in testicular vein samples compared to standard cubital vein measurement [37]. In a series of 80 blood samples from testicular cancer patients of which 11 were matched pairs before and after orchiectomy, miR 371-373 was particularly useful in patients without elevated of the standard markers AFP and hCGβ [38] . A similar study has also recently been published in a cohort of 30 testicular cancer patients and 18 healthy controls with a validation series of 59 cancer patients, claiming a better identification of testicular cancer patients than by AFP and hCGβ [39] . In another study miR-371-373 has been measured from cubital vein blood from 20 stage I testicular cancer patients and 17 healthy males together with the expression in 15 matching tumour specimens [40]. Measurement of the microRNA in blood thus seems to reflect the activity in tumour tissues. It is also promising that high expression of embryonal microRNAs, has been observed in tumour tissue from seminomas, a tumour type where the clinical used tumour markers (AFP and hCGβ) are not elevated [41,42]

Testicular tissues are exquisitely sensitive to the drug cisplatin, a phenomenon related to the formation of DNA adducts by the drug [43,44]. While it was previously held that repair of intrastrand crosslinks by the nucleotide excision repair (NER) proteins was a central mechanism, it seems now that the less common interstrand crosslink repair where among others the proteins ERCC1 and XPF are involved, seems more important for cisplatin sensitivity [45-48]. Similar finding has been reported in small clinical studies [46,47].

In most cancer types the TP53 gene coding for the tumour suppressing p53 protein , is mutated or deleted [49]. In contrast to this,TP53 is of wild type in majority of testicular cancer tissues, and p53 has likely increased nuclear expression [43,50,51]. This puzzle has therefore been intensely studied, but the role of the tumour suppressor p53 has been controversial [43]. The protein murine double minute 2 (Mdm2 and the more recently discovered MdmX) are induced downstream of p53 but contribute to inactivation of p53 by a negative feedback mechanism [52,53]. Some cell line studies indicated that this was the mechanism for inactivation of p53 also in testicular cancer. However, further studies in testicular cancer tissues revealed that most of the downstream activity of p53 was intact with the important exception of the expression of p21 which is transactivated by p53, actually was reduced [43,54,55]. Thus the lack of blocking of cell cycle progression by p21, which normally allows the tumour cell time to repair DNA damage, thereby favors apoptosis instead of repair. However, also other components of the apoptosis machinery have been implicated [56]. The mechanism of the defect expression of p21 protein as mentioned above seems to be inactivation of mRNA for p21 by the miR302 and miR371-373 families [24,29,57,58].

Several genome wide association studies (GWAS) have in genomic DNA identified several mutation markers associated with development of testicular cancer [59-61]. We and others have confirmed these associations and discovered some more [62-65]. Several markers,

especially associated with development and embryonal stem cells, are discovered in the different histological types of testicular cancer, but as yet not systematically assessed as prognostic or predictive factors in clinical series [66-68]. Recently data from exome sequencing of DNA from fresh testicular germ cell tumours from patients with bilateral cancer, indicate that each tumour develop independently [20].

Based on the data presented above, the project can be divided in the following tasks:''

Task 1. Determine miRs before and after orchiectomy with one sample before and three measurements the day after orchiectomy to determine the half life.

The hypothesis is that determination of level of miRs after orchiectomy can reliable confirm stage I in both seminomas and non-seminomas. Currently the final stage for non-seminomas is determined by a new full staging procedure with clinical assessment, imaging and measurement of the markers AFP, hCGβ and LDH after 6 -8 weeks of observation. Hopefully the patients therefore in the future can avoid the uncertainty imposed by the delay.

This will also be very helpful to diagnose stage IIA patients without elevated standard markers (typically for seminomas and teratomas), as these are followed by repeated staging procedures and delayed chemotherapy. By miRs it may be possible to reach a conclusion which exclude active disease or confirm possible disease activity which call for active treatment.

We aim at including at least 300 patients.

We will use Exiqon miRNome panels allowing identification of more than 700 different miRs to identify the specific miRs which are most reliable for further research. After identification of the most elevated microRNAS we will focus on these together with miR 371-373 and miR 302/367. They will be assessed by reverse transcription and analysis by real time PCR method.

Task 2. Use miRs to determine tumour response to chemotherapy and aid in decisions on treatment intensification or stopping therapy in testicular cancer patients with metastases..

In this project determination of the levels of miRs will be compared with measurement of AFP and hCGβ before and during treatment for metastases. The miRs will be particularly interesting in the 40% of snon-seminomas whch do not exhibit elevated tumour markers and where we currently have to assess the tumour shrinkage during follow up assessments by imaging modalities (CT or MRI), knowing that wat we see on an image not necessarily are viable tumour tissue and also that some tumours decrease slowly after therapy, especially seminomas.

We aim at including 100 patients with non-seminomas stage II-IV and 50seminoma stage II_IV.

Task 3. Assess the value of miRs to detect early recurrences after initial staging and therapy.

Currently we expect 10-20 % recurrences after therapy or in the group followed by observation (surveillance). Most of the recurrences are detectred by systematic measurement of tumour markers (AFP, hCGβ or LDH) and by imaging (Chest roentgenofram and MRI of abdomen/pelvix). Early detection of recurrences due to close monitoring of the patiens is one of the keys to the success of treatment of testicular cancer as most recurrences are now detected when still curable by chemotherapy. Most recurrences occur within 2 to 3 years from diagnosis.

This project will include 100 Seminoma stage I patients and 200 Non-seminoma stage I. As a separate group follow up of all patients will be followed for possible recurrence. We expect 10 -20 % recurrences depending on tumour type and stage, leaving 45 -90 patiens for assessment of the miRs role to early detect recurrences.

Task 4. Determine the clinical role of tissue markers in primary testicular cancer tissues.

From the patients mentioned above we will collect formalin embedded tumour blocks and perform *in situ hybridisation* with probes for the 5 best candidate miRs to prove that they are really produced by tumour tissues. Control will be adjacent normal testicular tissues, bearing in mind that cancer in situ lesions (intratesticular germ cell neoplasia) may express the markers. We plan to use double DIG-labeled miRCURY LNA microRNA detection probes, ISH buffer and reagents from Exiqon.

We aim at studying tumours from 200 patients

Task 5 Korrelation of markers detected with GWAS studies with the clinical presentation (stage) and prognosis (sespons to chemotherapy and recurrence)

From the parafin blocks we will select 4 tissue sylinders of 1 mm to be built into tissue microarrays (we use 4 in this project as some testiculer cancer are heterogenous and exhibit different morphological tissue types). In this project we will analyse protein expression of candidate proteins identified from genome wide association studies (GWAS) to examine if there is a relation to stage at presentation or as prognostic factors.

We will here include 300 tumour samples based on pevious experience with scoring immunohistochemical stained proteins in tumour tissues.

The study is open for testicular cancer patients who follow the SWENOTECA protocols in Norway and Sweden.

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