Minimal residual disease monitoring in neuroblastoma
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Chapter 8

General discussion
The research presented in this thesis focused on improving minimal residual disease detection in neuroblastoma and to further study the clinical significance of MRD monitoring.

Several retrospective studies have reported that MRD detection after or during induction therapy can be predictive of outcome (1-5). Our group has previously demonstrated in a retrospective study that early molecular BM clearance is associated with a better outcome. Viprey et al. demonstrated in their large prospective study that detection of neuroblastoma mRNA after completion of induction chemotherapy was associated with a poor outcome, however the level of mRNA at diagnosis was the strongest outcome predictor and neuroblastoma mRNA detection at end of induction did not have additive value to the predictive power of the level at diagnosis (6). In the interim analysis of our prospective study (chapter 7) high levels of BM infiltration at diagnosis were also associated with a poor outcome, whereas bone marrow (BM) clearance after 4 courses and at the end of induction chemotherapy was not associated with a better outcome. It should be mentioned that these conclusions are preliminary since they resulted from an interim analysis. In the study by Viprey et al. there is no difference in survival curves during the first 1.5 years after diagnosis and in the interim analysis of our study the follow up time is still relatively short. Furthermore, we were only able to assess the response in about half of the included patients, whereas neuroblastoma mRNA levels at diagnosis were available for most patients in the study. However, also our data indicate that high levels of neuroblastoma mRNA at diagnosis seem a more important prognostic factor than the response during induction chemotherapy. High levels of BM infiltration can also be detected by morphology or immunocytoLOGY and RQ-PCR techniques are not needed to detect these high levels. Indeed the level at diagnosis as determined by immunocytoLOGY has been described as prognostic factor (8).

As mentioned previously in retrospective studies a strong association between BM clearance and outcome was observed, however the two prospective studies failed to confirm this strong association. It is possible that there was some form of sampling bias in the retrospective studies. It is likely that in patients with a good clinical response no BM sampling was performed, whereas in patients with a poor response serial BM sampling was performed. This can lead to a large group of patients with a poor prognosis and most likely positive BM results. In the prospective studies BM samples were taken at fixed time points and much larger groups of patients were studied. Furthermore, patients were not uniformly treated in these retrospective studies. In the retrospective study performed by our group more than half of patients were treated with less intensive chemotherapy and some patients did not receive ASCT. The patients that received intensive chemotherapy may have had a better response and survival than the patients that received less intensive treatment. This might lead to differences in survival and response. Indeed in the retrospective study performed by our group 71% of patients had positive BM after 3
months of treatment and 41% after completion of induction chemotherapy, whereas in the interim analysis of our prospective study 60% had positive BM at the early time point and 35% after completion of induction chemotherapy. In all these studies from different groups different RNA markers and techniques for defining significant mRNA levels have been used (9-13). Consensus on the use of markers and techniques would facilitate the comparison of these results. At the moment international studies comparing results from different groups are ongoing.

It is interesting that when looking at the survival curves in the two prospective studies (Viprey et al and chapter 7), the majority of patients clearing their BM during or after completion of induction chemotherapy will suffer from recurrent disease. It is possible that the tumor levels in these patients are below the levels of detection, that the tumor cells causing the relapse are not present in BM or that these tumor cells escape clinical surveillance due to tumor heterogeneity and a low expression of the MRD markers. Cellular heterogeneity is an important aspect of many types of cancer. Epithelial to mesenchymal transition (EMT) has recently been demonstrated to generate cellular heterogeneity in neuroblastoma. In chapter 3, we identified a panel of markers specific for the detection of these mesenchymal (MES) neuroblastoma cells (cells that underwent EMT). These MES markers showed different dynamics than the classical neuro-epithelial (NE) markers and were more frequently detected in relapse patients. Also detection of these markers in PBSC samples was associated with an unfavorable outcome, whereas detection of NE MRD markers in PBSC samples failed to find an association with outcome (chapter 5)(14). These results suggest that these MES cells may be of importance in disease progression.

Ideally an MRD marker would be able to detect both MES and NE neuroblastoma cells. By using a combination of the “old” marker panel and the MES markers we would be able to detect NE and MES cells. Another option would be the use of DNA markers, as has been described in chapter 2 (15). We describe that DNA breakpoints used as MRD targets in neuroblastoma are reliable and stable markers. It is likely that NE and MES cells share the same genomic aberrations and that by using DNA markers both cell types can be detected. Another advantage of the use of these tumor-specific DNA markers is that there will be no amplification of normal hematological cells. In chapters 2 and 3 not enough clinical samples have been tested to investigate whether DNA markers or the combination of MES and NE markers indeed can identify additional positive samples and are clinically significant. To answer this question we should study these markers in large numbers of patients.

A second explanation for negative PCR results in patients with recurrent disease is that these neuroblastoma cells reside in other organs than the BM. Recurrence of high risk neuroblastoma can occur in BM in about 40%-70% of patients. In about 20% of patients a local recurrence occurs. Other sites for recurrent disease are bone, lymph nodes, liver,
lung and the central nervous system (CNS) (16, 17). Indeed Kramer et al. described that the CNS may be a sanctuary site for neuroblastoma and that with improvements in therapy there may be an increase in CNS relapses. Frequently in patients with relapses in the CNS this is the sole site of recurrence (18, 19). In chapter 4, we describe that ctDNA can originate from both metastases as primary tumor. Whether ctDNA can be used as a biomarker in patients relapsing in other organs than the BM is of interest and we are currently studying this. In our study we have used hypermethylated RASSF1A as marker, however this marker was occasionally found positive in healthy controls. Furthermore, RASSF1A is hypermethylated in all stage 4 tumors, but is variable in localized and stage 4s disease. Therefore, patient and tumor-specific techniques (mutations and aberrations) as described in chapter 3, may be used in the future as markers for the detection of ctDNA. The use of droplet digital PCR (ddPCR) may improve tumor DNA quantification and detection performance compared with RQ-PCR (20).

Recently Cheung et al. reported on the first prospective study investigating the clinical significance of MRD detection at a late time point during treatment, after 2 cycles of anti-GD2 immunotherapy. BM MRD before and after 2 cycles of immunotherapy was measured in a total of 319 patients, by using a four-marker panel (B4GALNT1, PHOX2B, CCND1 and ISL1). Detection of MRD was highly predictive of outcome after 2 cycles of immunotherapy. So this late response assessment could identify patients with increased risk of relapse or death and could help individualize therapy and identify patients that might benefit from alternative treatment options (21). The question arises why MRD detection at this late time point is a strong outcome predictor in contrast to MRD detection during and after completion of induction chemotherapy. The first explanation may be that there is a difference in the patient groups. Patients that received immunotherapy had no or minimal evidence of clinical disease at the start of immunotherapy and therefore the prognosis in this group of patients is better. When measuring MRD during induction chemotherapy also many patients with negative MRD results can suffer from relapse and the prognosis of the total group is poor. So it seems likely that it may be easier to identify patients with an increased risk of relapse in this group of patients with a better prognosis than in the patient group during induction chemotherapy. The second explanation may be the effect of treatment. It is likely that high dose chemotherapy and ASCT followed by immunotherapy will eradicate minimal residual disease. Therefore, it is possible that patients who still have MRD detected after induction chemotherapy, will become negative. Indeed as described by Cheung et al. MRD detection before the start of immunotherapy was not predictive of outcome, indicating that immunotherapy is capable of eliminating MRD. Since high-dose chemotherapy with autologous stem cell rescue has improved prognosis in high risk neuroblastoma patients (22), it is likely that this also will eradicate residual neuroblastoma cells.
RQ-PCR positivity in BM of localized neuroblastoma patients has been described in retrospective studies (23, 24). In chapter 6 we studied a large prospective cohort and frequently detected neuroblastoma mRNA at diagnosis, whereas very few systemic events occurred. However, detection of more than one marker (indicating higher levels of infiltration) was associated with an unfavorable outcome. Much larger studies are necessary to identify a group of patients with an increased risk of relapse in this cohort with good prognosis.

The high frequency of PCR positivity in this group of patients and the fact that we did not find an association with outcome in the whole group may be explained by the very low level of infiltration detected in many patients. It is possible that the mRNA originated from circulating and not infiltrating cells. Since it has been demonstrated that also extracellular vesicles can contain tumor specific mRNA (25, 26), this may be another possible source of low levels of mRNA in BM originating from the primary tumor and that the presence of this circulating mRNA has no predictive value. Our group is currently studying the presence of extracellular vesicles in neuroblastoma. Similar to ctDNA, by detecting neuroblastoma mRNA in vesicles in plasma of neuroblastoma patients, we may be able to detect the presence of tumor cells also in other organs than the BM and this might be a suitable tool for the monitoring of tumor burden in patients with localized disease.

**Summary**

Large prospective studies are ongoing or have been published concerning the clinical significance of minimal residual disease monitoring in neuroblastoma. Unfortunately to date there is no consensus yet in which markers and techniques for defining significant mRNA levels should be used. DNA markers are promising and good alternatives for RNA markers and should be studied alongside the RNA markers in large numbers of patients. Circulating tumor DNA may be a biomarker to assess response in patients with high risk neuroblastoma without BM disease or patients with recurrent disease in other organs than the BM.

Neuroblastoma mRNA levels in BM and blood at diagnosis and MRD detection after 2 cycles of anti-GD2 immunotherapy are strong outcome predictors. Whether early BM response provides additional prognostic value remains unclear at this moment. The different MRD monitoring studies should be completed and discussed by the different groups and if possible implemented in upcoming clinical trials. Furthermore, consensus on the use of markers and techniques would facilitate the comparison of these results. Overall the prognosis of high risk neuroblastoma patients is poor and also in the group of patients without MRD after 4 courses of treatment or after completion of induction
chemotherapy many patients suffer from recurrent disease. The current treatment is still not optimal and it therefore can be challenging to identify a group of patients with a good prognosis in these high risk patients. It is possible that the prognostic value of MRD detection during treatment will improve when alternative treatment options leading to improvements in survival become available.

**Recommendations**

- After completion of our prospective study we should compare the performance of the different markers and the techniques for defining significant mRNA levels with other groups. It is possible that the cut points determined by Viprey et al. will not be reproducible for our cohort, since patients were treated differently and therefore survival and response can be different. The reproducibility of these cut points in other cohorts may be a major disadvantage of using this technique for defining significant mRNA levels and should be studied. We should compare whether the high level at diagnosis identified as prognostic unfavorable by our group can identify the same patients as identified by the cut points in the study by Viprey et al. This way we may be able to compose the best panel of markers and techniques for defining significant mRNA levels. Also the prognostic value of the individual markers should be analyzed.
- Furthermore, we should internationally discuss the optimal time points for MRD detection and these time points should be implemented in clinical trials. For patients with high levels of neuroblastoma mRNA at diagnosis (ultra-high risk) current treatment is failing and these patients may benefit from alternative treatment strategies. MRD detection after 2 courses of immunotherapy identifies patients with increased risk of relapse and therefore MRD detection could help to individualize treatment. Whether early BM clearance during induction chemotherapy will provide additional prognostic information remains unclear at this moment.
- It is likely that also in our study the level of neuroblastoma mRNA at diagnosis is the most important prognostic factor and that BM clearance during induction therapy has no or limited additive value. Also many patients with negative MRD results during induction therapy may eventually suffer from recurrent disease. It is possible that MES mRNA markers or the combination of MES and NE markers are more important prognostic markers during induction therapy than NE markers alone.
- In future studies MES markers and DNA markers should be used alongside the NE markers. This may give more insight in the clinical significance of MES neuroblastoma cells. By using DNA markers or NE and MES RNA markers we will be able to detect both MES and NE cell types and may improve MRD detection and the predictive power of MRD detection during induction chemotherapy.
• However, it can be possible that patients with very early BM clearance (after 2 courses of chemotherapy) are patients with an extremely good response to treatment and might have a good prognosis. This has never been studied. In the final analysis of our prospective study this early time point should also be studied.

• If indeed MRD detection (by using the NE and MES markers and/or DNA markers) during induction therapy (early and late time points) does not provide additional prognostic value, RQ-PCR based BM testing should be limited to the time points at diagnosis and after two courses of immunotherapy. However, it is possible that with advances in treatment options, survival can improve and thereby also the prognostic value of MRD detection.

• In localized patients a second large prospective cohort should be studied and also BM minimal disease results should be compared with other factors known to influence event free survival in this group of patients (like gene expression signatures, the presence or absence of structural abnormalities and DNA ploidy)

• Detection of ctDNA is a suitable non-invasive tool for disease monitoring in neuroblastoma, highly correlates with disease status and is likely to originate from both primary tumor as metastases. Detection of ctDNA may be of additive value to monitor disease or detect relapse in patients without BM involvement. Therefore we should study the value of ctDNA detection in patients with local relapses and CNS relapses. Furthermore, also patient-specific mutations or aberrations may be used to detect ctDNA in neuroblastoma.
Reference List


