MicroRNA expression profiling of carcinomas of unknown origin

Loading samples into the HS 4800 Pro hybridization station in the service department at Exiqon

Around 5 % of all newly diagnosed metastatic cancers are carcinoma of unknown primary (CUP) origin, where the site of the primary tumor cannot be determined, despite the use of advanced immunohistochemical or radiological techniques. Because effective cancer treatment depends on early identification of the primary tumor, CUP patients have poor prognoses, with a median survival of 3-6 months and a one-year survival rate of less than 25 %.

Exiqon has developed a new molecular tool for the diagnosis of CUP, using microarray-based microRNA profiling data. MicroRNAs are good candidates for such a tool as their expression signatures can be used to classify specific cancers. Simultaneous detection of all microRNAs on an array presents a number of challenges. The short length (21-23 nt) of microRNAs leaves little room for probe optimization, and many microRNAs are very similar in sequence, varying by only one or two bases. Also, microRNAs possess huge variation in base composition, which results in a large T_M range of microRNA-DNA probe dimers (Figure 1, gray bars). To overcome these challenges, the capture probes used in the miRCURY™ LNA™ microRNA Arrays incorporate Exiqon's high affinity Locked Nucleic Acid (LNA) technology. The T_M-normalized probes (Figure 1, yellow bars) of these arrays result in unparalleled sensitivity and excellent mismatch discrimination for all microRNAs, making them superior to DNA probes (Figure 2) for efficient discrimination between closely related microRNA family members and microRNA profiling from as low as 30 ng total RNA.

Automation is an important factor for standardization of complex experimental procedures, such as those involved in hybridization experiments, to ensure reproducible and reliable data. Tecan's HS Pro series of hybridization stations – available as HS 400™ Pro, which handles up to four slides, and a larger version, HS 4800™ Pro, which can process up to 48 slides – enables full automation of microarray hybridization experiments with minimal handling of solutions and slides. The hybridization station applies washing buffer to the active slide surface within a hybridization chamber that seals the slide from the top to improve washing efficiency. During hybridization, the sample is subjected to the patented agitation mechanism to guarantee uniform incubation. The agitation, together with hybridization station's unique active bubble suppression (ABS™) system, helps to achieve maximum specificity, sensitivity and reproducibility of results. The on-slide nitrogen drying procedure (OSND™) results in a low and uniform background, and the slides can be scanned immediately without the need for tedious drying procedures.

Exiqon developed the miRCURY LNA microRNA Arrays using the HS 4800 Pro, and three are in its R&D laboratories for product development. Another seven HS 4800 Pros are being used in the service department and are operated to full capacity, every day, where samples received from customers are processed. Although Exiqon's arrays can be hybridized manually, the Company has always recommended automated processing using Tecan's hybridization stations for the most reliable results.

To develop a classifier tool for diagnosis of CUP, Exiqon's miRCURY LNA microRNA Arrays were processed on Tecan HS 4800 Pro hybridization station, for microRNA profiling of normal and tumor tissues. More than 500 tumor and normal adjacent tissue samples were collected from both fresh frozen and formalin fixed, paraffin embedded sections of 18 tissue types (adrenal, bladder, breast, cervix, colon, esophagus, gall bladder, kidney, liver, lung, ovary, pancreas, prostate, rectum, small intestine, stomach, testis and uterus), representing the most common tissues of origin for CUP. Total RNA from tissue was analyzed for microRNA expression on the

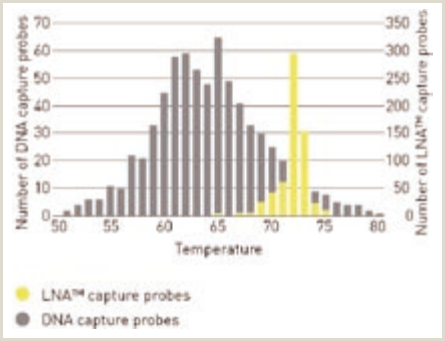

Figure 1. $_{TM}$-normalized LNA capture probes. DNA capture probes (gray bars) for human microRNAs have a $_{TM}$ range of more than 30 °C and an average $_{TM}$ of 64 °C (StDev = 5 °C). The LNA capture probes (yellow bars) have a $_{TM}$ range of only 10.0 °C and an average $_{TM}$ of 71.5 °C (StDev = 1.6 °C).

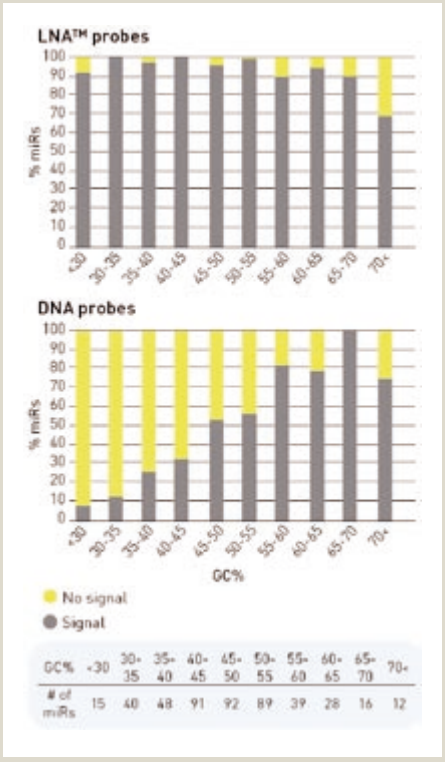

Figure 2. LNA-based arrays are superior to DNA-based arrays at detecting microRNA. Upper panel: Exiqon's LNA-based array. Lower panel: A DNA-based competitor array. Gray bars indicate the percentage of capture probes capable of detecting 50 amol of synthetic microRNA within the given range of GC content. Yellow bars indicate the percentage of capture probes not detected at 50 amol. It is clear that DNA-based arrays show decreasing efficiency with decreasing GC content.

miRCURY LNA microRNA Array using the HS 4800 Pro hybridization station, and a unique microRNA profile for each of the 18 tissue types was generated (the full protocol for hybridization and washing can be downloaded at www.tecan.com or at www.exiqon.com). A microRNA expression database was established, and a detailed analysis of these results revealed that a cancer classifier based on the expression levels of seven microRNAs is sufficient to distinguish between tumors from the 18 tissue types.

The classifier tool was subsequently used to identify the tumor type of a patient suffering from CUP by analyzing a biopsy from a lymph node metastasis. A biopsy from a lymph node metastasis from a patient suffering from CUP was profiled on the miRCURY LNA microRNA Array. The microRNA expression level of the metastasis was compared to the average expression level across the 18 tissues and, based on a simple cluster analysis, the lymph node metastasis microRNA profile was shown to belong to the colorectal microRNA cluster (Figure 3). A primary colon tumor was later found in this patient, which highlighted the potential of this method as a tool for identifying primary tumors in CUP patients. Therefore, in this case, one would – based on the microRNA profile alone – be able to direct the treatment against a colon cancer without having to perform a full body PET scan to search for the primary tumor. A colonoscopy would have been sufficient to verify this result.

Taken together, the results indicated that the miRCURY LNA Array used in conjunction with the HS 4800 Pro hybridization station is a very powerful combination for high quality microRNA expression studies. It permits highly specific probe binding by yielding maximum sensitivity and a high inter- and intra-slide reproducibility by circumventing the handling drawbacks and the decrease in data quality associated with manual hybridization experiments.

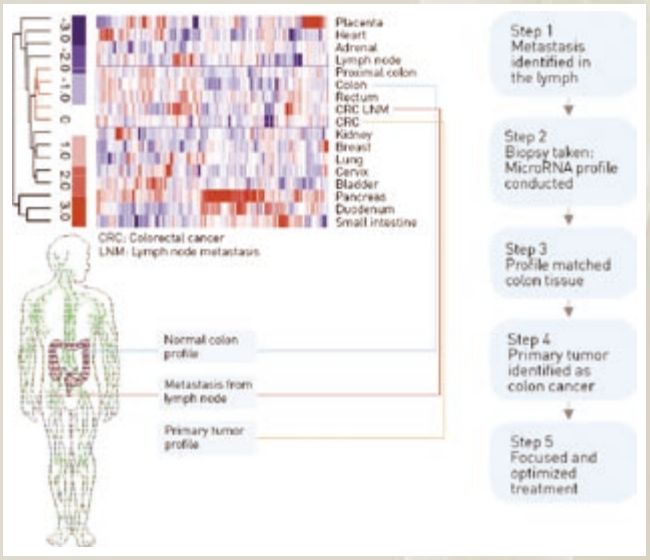

Figure 3. The workflow and result of the identification of the origin of a primary cancer. The cluster analysis identified the lymph node metastasis microRNA profile as colorectal cancer. All the colorectal samples, including the metastasis, cluster together and clearly indicate that the colon was the origin of the metastasis.