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Quality of Biological Samples

The increasing popularity of microarray analysis has been partially fueled by the frustration of biologists with limited technological tools they have had at their disposal for gaining a comprehensive understanding of complex biological problems. The completion of the Human Genome Project and the understanding it heralded threw into even greater relief the problems with the current technology. Although still not finally known, the number of genes in the human genome is estimated to exceed 40,000. These genes and their protein products determine biological phenotypes, both normal and abnormal. Traditional molecular biology methods have only allowed biologists to peek at the activities of a small number of genes that are associated with certain biological processes. In addition, although the reductionist method has enabled researchers to delineate a number of signal transduction pathways, it cannot yield a comprehensive picture of the systems under study. By allowing simultaneous measurement of the expression of thousands of genes, microarray technologies have marked a defining moment in biological research. Indeed, many publications have now shown the power of gene expression profiling in the diagnosis and prognosis of disease and the identification of gene targets. Microarray analysis is rapidly becoming a standard research tool.

It is therefore important for those outside the small pool of researchers who now do microarray analysis to know not only how it is done, but how to do it themselves. In general, a microarray experiment starts with the acquisition of biological materials from which RNA is isolated. For experiments involving established and relatively homogeneous cell lines, this is quite straightforward. However, for many experiments involving clinical tissues, the process is more complex and special attention must be paid to quality control. Because a

central purpose of most microarray experiments is to map gene expression in biological samples, this chapter focuses on the quality control issues related to the subjects of experimentation—the tissues.

1.1 TISSUE ACQUISITION, HANDLING, AND STORAGE

In our expression microarray core facility, we have found that the best way to get good results from a microarray experiment is to start with RNA of exceptionally high quality. Isolating such RNA poses a significant problem, however, especially when the samples are taken directly from patients. However, this obstacle must be overcome because patient samples, as opposed to cell lines or animal models, tell the most physiologically relevant story.

Because RNA is not stable and degrades quickly if tissues are not handled and stored correctly, the method of acquiring, handling, and storing fresh tissues obtained during surgery for microarray analysis is critical to the success of the experiments. Some frequently encountered problems and possible consequences include:

Problem 1. Tissues are removed from patients by surgeons and placed on a counter, where they remain for hours before they are processed. Because the blood flow to the tissue is stopped, the cells start to undergo apoptosis, and RNase is released, which chews away at the RNA in the decaying cells.

Problem 2. Blood is drawn from patients and put into collection tubes, which are kept at room temperature for hours or overnight before they are transferred to the research laboratories. During this time, because the cells are under a different physiological condition and temperature, some cells begin to die, RNA begins to degrade, and gene expression begins to alter.

Problem 3. Tissues are cut into pieces and fixed in formalin. However, RNA is not stable in formalin and becomes degraded.

These problems highlight the importance of the active participation of clinical personnel in clinical research. To have the best tissues with good-quality RNA that accurately reflects the transcriptome in its physiological state, surgeons, pathologists, research nurses, and tissue bank personnel should know and strictly follow the correct tissue acquisition, handling, and storage procedures. First, tissues and blood samples should be kept at room temperature only for a brief time, preferably less than 30 minutes. After this, the tissues or blood cells should either be snap-frozen in liquid nitrogen or be put into solutions that inhibit RNase, with RNAlater—the most common RNase-inhibiting reagent. In the latter case, tissues should be cut into small pieces and immersed in the RNAlater, which can then penetrate the tissues and inhibit the RNase. Tissues can be maintained in this solution for a few weeks

at 4°C. For long-term storage, however, tissues should be transferred to another tube and stored in a −80°C freezer. Alternatively, the tissues can be cut into small pieces, transferred to tubes that can be submerged in liquid nitrogen for snap-freezing, and stored in a −80°C freezer. Generally, tissues that are handled and stored in this way produce good-quality RNA. We prefer RNAlater to liquid nitrogen, however, because it is much more practical for research nurses in the operating room to put tissues into a tube prefilled with RNAlater than to prepare them in liquid nitrogen. It is also safer, in that liquid nitrogen poses a hazard in a patient-care setting.

The freezing solution is also important. For example, one that is used in the neuropathology laboratory at the University of Texas M. D. Anderson Cancer Center is the Tissue-Tek OCT Compound (Sakura/Tissue-Tek Company, Torrance, California), which is an inert whitish solution that helps maintain the morphology of the tissues during the cutting of tissues for slides. It consists of 10.24% polyvinyl alcohol, 4.26% polyethylene glycol, and 85.50% inert ingredients. Tissues stored in this solution should also be frozen as quickly as possible. OCT stands for optimal cutting temperature, and as the name implies, the solution was originally formulated to facilitate the cryostat sectioning of fresh frozen tissue (i.e., frozen section preparation). OCT has, however, also been proven to work well for tissue storage. It came to be used because, to make an intraoperative diagnosis, tissue can be embedded in OCT rather than paraffin (as is done in most hospitals), and then these OCT-embedded tissues can be frozen and stored. A second reason is that pathologists typically embed all of the extra tissue destined for the tumor bank in OCT solution because they want to be able to screen every block to determine, for example, whether the content is necrotic or pure, and the only way to cut sections for this is to embed the tissue in OCT. Such screening is not necessary with some tumors (e.g., meningiomas) that are clearly pure, even grossly. However, this would not be the case for gliomas because of the diffuse infiltration that may be present. Of course, one can flash-freeze tissue directly in liquid nitrogen, but then one cannot use such material to cut tissue sections. Some laboratories do freeze tissue, even glioma tissue, directly in liquid nitrogen and perform experiments with it, simply assuming that the tumors are pure, but assumption is a dangerous business in science because it can lead to erroneous conclusions, and therefore this practice is not recommended.

In the next section we discuss the importance of pathological evaluation. Before that, however, we describe those tissue collection and storage methods that yield the most optimal RNA quality. This is especially important for biopsy tissue and fine-needle aspirates, which are very limited in quantity.

In a study carried out in our laboratory, we compared the RNA extracted from tissue biopsy specimens that were prepared in four different ways: (1) snap-frozen in liquid nitrogen; (2) preserved in RNAlater; (3) preserved in RNAlater, fixed in ethanol, and embedded in OCT; and (4) preserved in RNAlater, fixed in ethanol, and embedded in paraffin. In this experiment,

normal and tumor tissue samples were obtained in a standard clinical manner by surgical resection from seven patients with colon cancer. Each tissue sample was divided into four portions and subjected to one of the four study conditions, all according to standard hospital procedure: The paraffin-embedded samples were stored at room temperature until the RNA was extracted, whereas the other three were stored at −80°C until the RNA was extracted.

Each RNA sample was analyzed with a spectrophotometer at 260, 280, and 230 nm to detect protein and polysaccharide contamination and determine the RNA concentration. RNA degradation and genomic DNA contamination were assessed in aliquots of the preparations by electrophoresis on a 1% denaturing agarose gel containing 1X MOPS (morpholinopropanesulfonic acid) buffer and 2% formaldehyde. Each sample was also loaded into an RNA LabChip (Caliper Technologies Corp., Mountain View, California) and analyzed on the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, California) using the RNA 6000 Nano Assay.

Two representative RNA samples were also used to hybridize to a cDNA microarray containing 4800 features produced in-house. Good microarray results were obtained for all but the samples embedded in paraffin, indicating that the RNA isolated from the biopsy samples prepared under the other three conditions was of good quality for microarray analysis. Nonetheless, for the reasons given earlier, we prefer RNAlater to liquid nitrogen as a processing agent.

Clinical samples obtained by fine-needle aspiration (FNA) pose a unique challenge, however. Unlike biopsy specimens, in which cells are in a lump of tissues, tissues obtained by FNA are often broken up, and when they are immersed in RNAlater, the cells are often dispersed. FNA samples from tumors are particularly useful, however, because they represent a relatively pure population of tumor cells that is relatively free of the contaminating normal cells found in a surgical resection specimen. This purity is particularly important when one considers the subtle changes that may occur at the beginning of tumorigenesis. For example, contaminating normal cells could obscure a small but important change in a tumor's gene expression pattern. FNA specimens are also preferable because FNA is much safer and less invasive than biopsy or surgery. FNA could also enable repeated aspirates to be collected from the same tumor so that the progression of the disease and the tumor's response to therapy could be monitored. It is therefore important to be able to use FNA samples for microarray analyses because of the valuable potential they hold for gaining insight into the molecular details and prognostic indicators for certain types of cancer for which there is a limited volume of relatively pure tumor cells. Indeed, such clinical samples can be used with high efficiency in high-density expression microarray analyses. For this reason, it is essential to retrieve as much high-quality genetic material as possible from each FNA sample.

We thus also evaluated RNA quality and quantity in FNA specimens and found that because the cells are partially lysed, up to 50% of the RNA in some specimens is released from the cells into the RNAlater solution and thus lost, which is quite significant for FNA samples. In most protocols involving the use of RNAlater, tissues are separated from the RNAlater solution by centrifugation and then the pellet is exposed to an RNA isolation solution. To minimize the loss of RNA, we suggest keeping the RNAlater solution portion and also recovering the RNA from the RNAlater solution. To determine the feasibility of doing this, we used RNA isolated from the RNAlater supernatant and RNA isolated from the FNA pellet for microarrays and found that the results from both were very concordant. Thus, excessive RNAlater should not be added to the FNA tissue at the acquisition step. Two hundred microliters should be sufficient. FNA samples, therefore, yield sufficient RNA of adequate quality for microarray analysis, qualities that together allow for valuable high-density analysis.

1.2 PATHOLOGICAL EVALUATION

A common problem with microarray experiments involving the use of clinical samples is that a pathological evaluation of the tissues is not done before the experiments. This can lead to flawed results. We will illustrate this problem using two experiences we have had in our laboratory. The first example comes from a genomic study of sarcomas. Sarcoma is a cancer of soft tissues. Leiomyosarcoma is a type of sarcoma of muscle origin that often grows in the gastrointestinal tract for a long time before being discovered. Some of these weigh 20 pounds (9 kg) or more when they are finally removed during surgery. To remove most of the tumor that may have infiltrated into neighboring normal tissues, surrounding normal tissues, including the stomach, are also routinely removed. The tumors are then cut into small pieces for tissue banking, some of which are used for research purposes. The normal tissues removed during surgery are used as controls. It is, therefore, not difficult to envision that sometimes the pieces labeled "tumor" may actually contain a large fraction of normal tissue. In the absence of a pathological evaluation of the tissues, the true nature of the tissue would not be known. Similarly, normal stomach tissues can consist of either muscle cells or epithelial cells, depending on where the cut is made. Since muscle tissues are the corresponding normal tissues for leiomyosarcomas, a tissue sample composed mainly of epithelial cells would not be the correct control material for a leiomyosarcoma. Pathological evaluation would ensure that the correct normal tissues are used as the control material.

The second example comes from a study in which we were using specimens from breast cancer lymph node metastases. Out of the 18 samples removed at surgery, pathological examination revealed that eight had infiltrating lymphocytes that constituted more than 50% of the cell population. Thus, these

samples were not used for the subsequent microarray study. If we had not performed this evaluation and used those tissues in the study, although the number of specimens in the study would have been higher, the results would have been highly confounded because of the presence of a large percentage of nontumor cells.

A third problem that can arise is errors in pathological classification and database tracking. It is commonly known by pathologists that many tumors have atypical morphologies and categorizing these cases sometimes involves guesswork. Different pathologists may even disagree on a diagnosis. Honest mistakes can also occur and sometimes records get mixed up. Thus, it is always prudent to reevaluate the tissues to be used in a molecular study to confirm that they are what they are supposed to be. There are also stories of highly regarded molecular classification studies using microarray analysis that turn out to be flawed due to problems of misclassification resulting from misdiagnosis during the pathological reevaluation of the material.

When molecular and genomic biologists first enter the world of pathology, they typically are amazed at the complexity of tissues—a world far different from that of pure tumor cells in a culture dish, which laboratory researchers typically enjoy. What pathologists see is quite a frustratingly heterogeneous picture of various cell populations. We describe some of these real-world experiences here.

1.3 TISSUE HETEROGENEITY AND LASER CAPTURE MICRODISSECTION

The view of cancer researchers used to working with cancer cell lines can be very simplistic. They often view cancers as being a large group of cancer cells growing together. However, this is far from reality. *In vivo* tissues, whether normal or diseased, are very heterogeneous. For example, in blood, there are cells of different lineages: the highly specialized red blood cells, which transport oxygen to the body; the lymphoid cells (B and T cells), which are important to the immune response; and the myeloid cells (monocytes, granulocytes, and macrophages), which are important to the inflammatory response, among other functions. In breast tissues, there are ductal epithelial cells, stromal cells such as fibroblasts, endothelial cells that make up the blood vessels, fat cells, and immune cells such as lymphocytes. Even in the *immune-privileged brain*, there are neurons as well as several different types of glial cells, such as astrocytes, oligodendrocytes, and microglial cells. Because of the heterogeneous nature of normal cells, therefore, it is often difficult to use normal tissues as "normal" control for comparison studies. For example, unless the normal astrocytes are microdissected, normal brain tissues, which contain many neurons, are not a suitable normal control material for astrocytomas. In leukemia studies, to serve as a normal control for B-cell ma-

lignancies, normal cell populations such as B cells can be sorted out by flow cytometry [or fluorescent-activated cell sorting (FACS)] using B-cell-specific surface markers. Cell surface markers in combination with FACS sorting can also be used to purify many other cell types, such as endothelial cells.

In cancer, a major area of research in which microarray studies are used, the tissues obtained at surgery can also be highly heterogeneous, although the core of the tumors may be rich in cancer cells. These heterogeneities are manifested at several levels. The first level of heterogeneity may cause the most artifacts if quality control is not observed. Because most tissues are obtained during surgical removal and to ensure a near-complete resection of the tumor and invasive tumor cells, surrounding normal tissues are also routinely removed, most tumor tissue specimens have contaminating normal tissues. A particular tissue block used for research may also have no tumor tissue, or different blocks may have different percentages of tumor cells. From this, one can see that obtaining an accurate tumor-specific gene expression signature strongly depends on the quality of the specimens. It is therefore extremely important to have the piece of tissue evaluated by a pathologist before processing it for microarray experiments. In such an evaluation, pathologists normally cut a section from the top and the bottom of the tissue specimen and evaluate the tumor makeup after routine H&E staining. Some trimming may then be done to remove contaminating normal tissues.

The second level of heterogeneity is biological. For example, in the case of a human glioma, pathological evaluation of the tissues often reveals mixed morphological features. As shown in Figure 1.1, panels A to D, four microscopic fields from the same glioblastoma show small cell (A), spindle cell (B), giant cell (C), and epithelioid (D) differentiation. In other tissues, all these features may be intermingled. Different tissues can also have different amounts of vascularization. In particular, low-grade tumors typically have low levels of angiogenesis, and high-grade tumors have high levels of angiogenesis. Therefore, a difference in the gene expression signature between different tumor tissues may imply different things, one of which could be a difference in the amount of endothelial cells in the tumor.

Even when tissues look homogeneous in their morphology, different cells in the tissues may not behave the same at the molecular level. This is best illustrated by immunohistochemistry studies using different molecular markers. To use astrocytomas again as an example, two different areas of the same oligodendroglioma show strikingly different proliferation activities, indicated by staining of MIB-1 marker (Figure 1.1, panels E and F). Another example is that the glial cell marker glial fibrillary acidic protein (GFAP) can have variable levels of expression in different areas of one glioblastoma (Figure 1.1, panels G and H). This is also the case for many other markers in different subsets of tumor cells in some localized region.

A further consideration is that tumor tissues such as colon and breast cancers are often significantly intermingled with stromal cells and infiltrated with inflammatory cells and lymphocytes. As shown in panels A to H in Figure

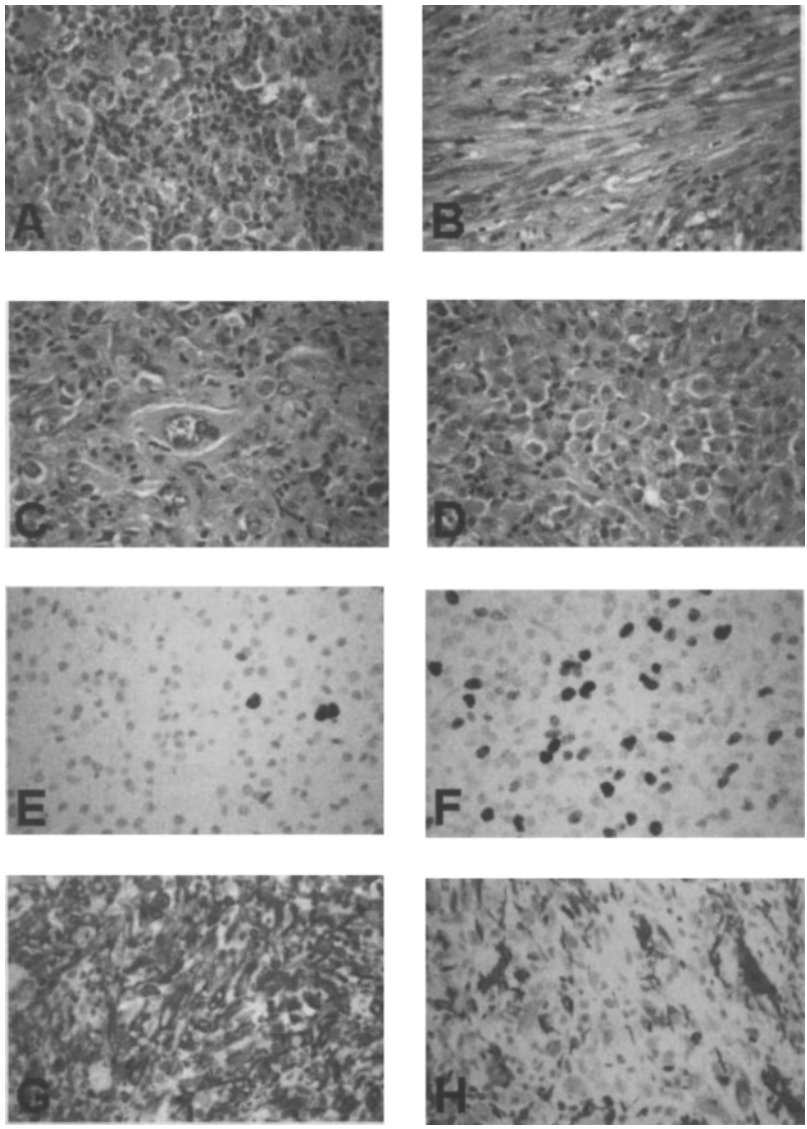

Fig. 1.1 Phenotypic, proliferative, and molecular heterogeneity in brain tumors. Phenotypic heterogeneity (A–D): Four microscopic fields from the same glioblastoma show small cell (A), spindle cell (B), giant cell (C), and epithelioid (D) differentiation. (H&E, 200×.) Proliferative heterogeneity (E–F): Two different areas of the same oligodendroglioma show strikingly different proliferation activities. (MIB-1 immunocytochemistry; 200×.) Molecular heterogeneity (G–H): Two areas of one glioblastoma illustrate variable GFAP gene expression. (GFAP immunocytochemistry; 200×). (This figure was generously provided by Dr. Gregory N. Fuller, The University of Texas M. D. Anderson Cancer Center.) See insert for a color representation of this figure.

1.2, highly morphologically different colon cancer cells display invasion into stromal cells and adipose tissues and are infiltrated with lymphocytes. Similarly, in breast cancer tissues, different parts of the same tumor may have very different cell populations. Some tumor specimens are composed mostly of cancer cells (Figure 1.3, panel A), whereas other tumor specimens have less than 50% of tumor cells (Figure 1.3, panel B). Different breast metastatic tumors in the lymph nodes can have drastically different amounts of infiltrating lymphocytes. Therefore, a comparison of the gene expression of different breast cancer tissues could ultimately show only the difference between breast cancer cells and lymphocytes should tumor-rich and lymphocyte-rich tissues be compared.

These examples point out the heterogeneous nature of the tissues we study and underscore the impact that different populations of cells in a tissue can have on the final microarray results, which only reflect the population average. Obviously, pathological evaluation is an important quality control step when selecting suitable tissues for a particular study. Laser capture microdissection technology was developed as a means of further overcoming this problem. In this technology, cells of interest are dissected from the tissues for study. However, although this technology has been extremely successful in isolating cells from paraffin-embedded tissues for DNA-based assays, frozen tissues have to be used for RNA-based assays such as microarrays, and a key limiting factor is isolating a sufficient amount of cells so that there is enough RNA for the microarray study. Nonetheless, with the development of amplification protocols for microarray analyses (discussed in Chapter 3), this technology is now more feasible and its popularity will probably surge in the coming years.

A drawback of the selection of cells by microdissection, however, is that it is based on subjective standards such as cell morphology, but morphologically similar cells may not be genotypically similar. To obtain an accurate picture, therefore, one may need to perform single cell-based assays. However, this then takes us away from the systems point of view of biology. In addition, this may not be practical because we do not know how many single cells we need to examine before we can draw a conclusion about the tissues. Therefore, there needs to be a balance between the reductionist and systems approaches. In other words, we need to remove those things that will interfere with the systems under study or the goals of the study, but not those things that are part of the systems. For example, angiogenesis and inflammation are part of cancer systems. Indeed, recent studies have shown that tumor cells often recruit inflammatory cells and make them coconspirators in tumorigenesis by inducing them to secrete proteins such as metalloproteinase, which facilitate tumor cell invasion and metastasis. Obviously, microdissection would remove these important cancer factors from the systems.

The intimate cross-talk between cancer cells and noncancer cells in cancer tissues is also illustrated by one of our recent studies (Kobayashi *et al.*, 2003). Diffuse large B-cell lymphoma (DLBCL) is a very heterogeneous disease with different surface markers expressed in the cells of different patients. One

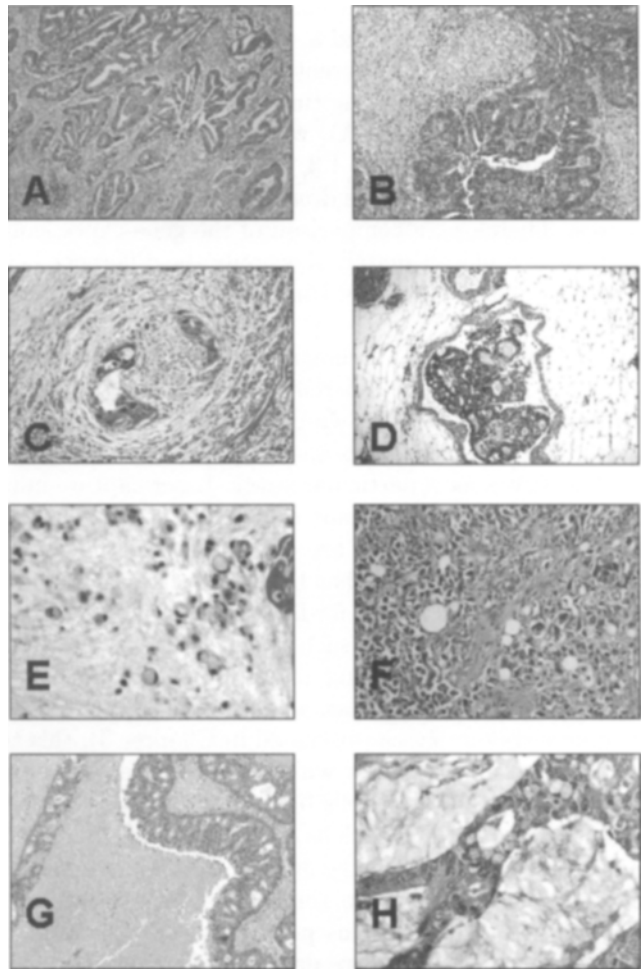

Fig. 1.2 Phenotypic heterogeneity in colon tumors. Moderately differentiated adenocarcinoma of colon (A–D): Elongated malignant glands arranged in a corkscrew pattern invade into the stroma (A). A different tumor displays a cribriform pattern and a prominent periglandular lymphocytic infiltrate (B). (H&E, 200×.) Same tumor focally invades the perineurium (C) and is present in the vascular space (D). (H&E, 400×.) Poorly differentiated adenocarcinoma of colon (E–F): Discohesive signet-ring cells floating in pools of mucin (E). (H&E, 400×.) Highly anaplastic tumor with pleomorphic cells, invading into the pericolonic adipose tissue (F). (H&E, 200×.) Adenocarcinoma of colon with ribbonlike pattern (G–H): Enlarged, dilated malignant glands with central necrosis (G). (H&E, 200×.) Mucinous tumor with pleomorphic cells and abundant extracellular mucin (H). (H&E, 400×.) (This figure was generously provided by Dr. Lucian Chirieac, The University of Texas M. D. Anderson Cancer Center.) See insert for a color representation of this figure.

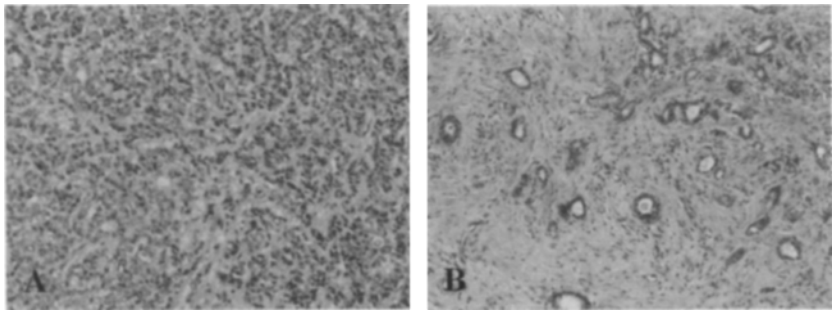

Fig. 1.3 The histopathological appearance is variable among different invasive breast cancers. Some cancers consist of an almost pure population of breast cancer cells (A). Most cancers contain a significant component of desmoplastic stroma (B) that is active in angiogenesis, fibrosis, myocontractility, and immunity. (This figure was generously provided by Dr. Fraser Symmans, The University of Texas M. D. Anderson Cancer Center.) See insert for a color representation of this figure.

group (10% of patients with DLBCL) is positive for the CD5 marker and has a poorer overall prognosis than patients with CD5-negative DLBCL. We carried out a microarray study to characterize the gene expression activities of CD5-positive and CD5-negative DLBCL. Among the differentially expressed genes were integrin beta 1 and CD36. Immunohistochemical staining then showed that integrin beta 1 indeed is expressed in most CD5-positive DLBCLs, which is consistent with the greater invasiveness of this subtype of DLBCL. The staining for CD36 also showed some striking patterns. CD36 was expressed in CD5-positive DLBCLs, but only in the endothelial cells of large vessels. In contrast, CD36 was not expressed in CD5-negative DLBCLs, although the large vessels were present (Figure 1.4). This is a good example of normal cells in different cancer tissues expressing different genes, which very likely contributes to the different biological behavior of the tumors. Microdissection of only the tumor cells for study would have missed this potentially important information. One solution, of course, would be to microdissect the vessel cells to obtain vessel-specific biological information as well.

1.4 SUMMARY

It is important to appreciate tissue heterogeneity and its effect on the results of microarray analyses. As a quality control step, it is worthwhile to have the active participation of pathologists to evaluate the cellular composition of the tissues under study. Depending on the goals of a study, one then has to decide how much tissue and cell purification to do, either by coarse-

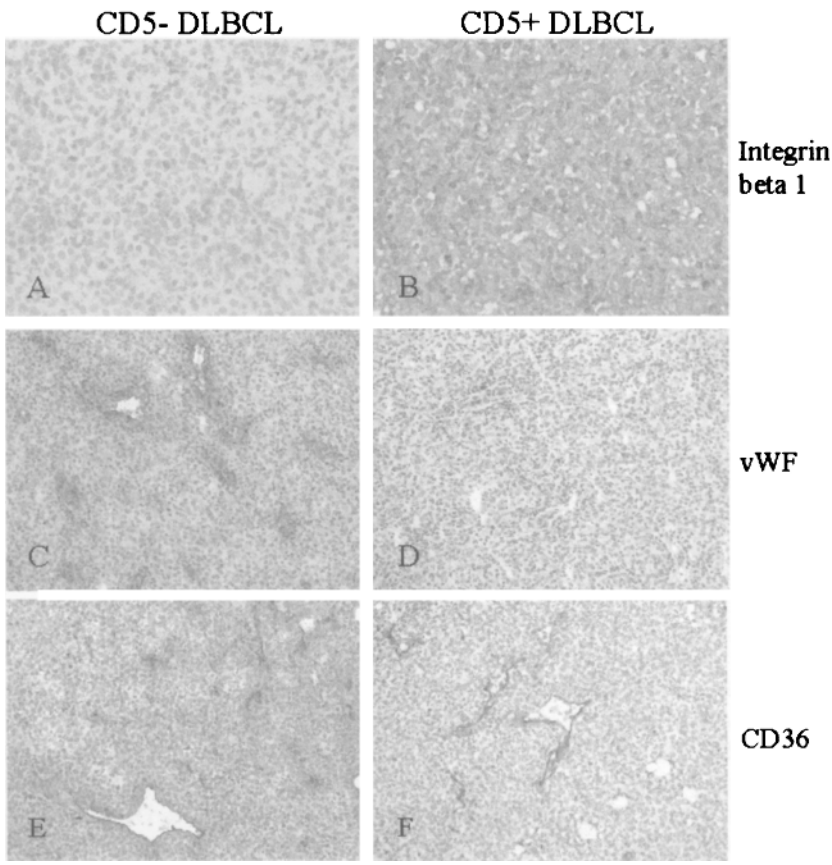

Fig. 1.4 Immunohistochemical staining of lymphoma tissues for integrin beta 1, vWF, and CD36. Integrin beta 1 was weakly stained for CD5-negative DLBCL (A) and strongly stained for CD5-positive DLBCL (B). vWF was stained strongly in the vascular cells of both CD5-negative and CD5-positive DLBCL (C and D). CD36 was not stained in CD5-negative DLBCL (E) and stained highly in the vascular cells of CD5-positive DLBCL (F). See insert for a color representation of this figure.

scale trimming or laser capture microdissection. The results of population-average studies eventually need to be validated by cell-based assays, such as immunohistochemistry analyses to gain spatial information on gene activities.

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