Comments from well recognized experts in the field

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Sick lobules within the sick lobe?

Breast is a glandular organ with lobar morphology. The median number of the lobes within a female breast is 27 **[1]**, a number showing wide variations. The lobes also vary in their size and shape and may comprise everything between 1% and 25% of the breast volume **[2]**. Most of the lobes are several centimeter in size and contains a lactiferous duct with its branches which end up in hundreds and thousands of lobules, and the corresponding stroma. The lobules are about millimeter large structures comprising acini, the intralobular part of the terminal duct, and the special intralobular stroma.

There is an increasing body of morphological evidence that breast cancer develops in one of the breast lobes in vast majority of the cases leaving the other lobes free of the disease **[3]**. Even if the *in situ* foci are multifocal with gaps of "normal" structures in between, they seem to belong to the same ductal system, the same lobe. The microscopically normal tissue may be genetically abnormal and show identical genetic changes with malignant cells in the histologically obvious cancer. These morphological and genetic changes may occupy a several centimeter large area within the breast and represent additional evidence for a risk tissue being limited to a contiguous space within the breast [**3]**. In our concept, this risk tissue corresponds to a "sick" lobe, a lobe that contains numerous progenitor cells and/or progenitor cells that are more sensitive to exogenous or endogenous oncogenic stimuli compared to the progenitor cells in the other "healthy" lobes **[4]**.

The classical concept of Wellings states that cancer develops within a lobule (terminal ductal-lobular unit) from where the cancer cells spread to the ducts and other lobules. There is growing evidence for the contrary, namely that cancer may develop in any part of a lobe: within the lobules, within the ducts or in both, in several lobules simultaneously or with considerable time difference, within one or several segments of the same lobe or within large parts or the entire lobe at the same time. This variety of the biological timing of malignant transformation explains the morphological heterogeneity of breast cancer already at the early stage of its development **[5]**.

During the last years, Dr Man and his research team has provided important pieces of evidence for malignant alteration being detectable in morphologically normal cells in breast and in prostate. In my view, the body of evidence presented in Dr. Man's paper in the present issue of the Journal of Cancer identifies partially transformed progenitor cells occupying a large volume of morphologically normal breast tissue. In contrast to our research approach focusing on subgross architecture, they focus on subtle microscopic details, but our results are congruent and facilitate the possibility of breast cancer developing in an area of the tissue rather than on a single cell level. Their further findings indicate certain interactions of these partly transformed cells and their altered microenvironment being sufficient to result in invasion without previous development of morphologically malignant structures. These findings are so surprising that they warrant further intensive research, but if confirmed, they may generally change the view of carcinogenesis.

I have previously raised the idea of possible metastatic spread of morphologically non-malignant partially transformed progenitor cells developing metastatic deposits prior to or without appearance of a cancer on primary location, stating the following: "Mutant stem cells and committed progenitor cells share many characteristics with malignant stem cells. These mobile cells may, in some cases, enter the circulation and be transported to lymph nodes and other organs. Malignant transformation of the cell progeny may require several years or decades, like their counterparts within the breast. Transformation of these relocated mutant progenitor cells prior to the transformation of the intramammary compartment of these cells may give rise to a metastasis of "unknown origin". This concept may represent a possible explanation for, at least some cases of, the so-called CUP (cancer with unknown primary) syndrome. Such a transformation may also take place decades later than the intramammary transformation." **[3]**. My idea expressed above was only a hypothesis without any

evidence. The results presented here carries a piece of evidence and may represent a possible explanation for the CUP syndrome.

Thus, I share the views of Dr. Man et al on several points. Our approach on architectural level and their approach on cellular level may fruitfully complete each other in explaining the earliest changes in breast carcinogenesis.

References

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Thank you so much for the very interesting paper and the opportunity to comment. I have read through your paper carefully and have made some changes using comment in the text itself. Below I will list the main points here:

1. This is a potentially very important observation that impacts our theory of cancer. It is therefore important to be thorough and include some control adjacent tissues that are not in clusters or lobules to make sure that they are not positive for malignancy-associated changes you observed in clusters. Also, you may want to examine other cancers in addition to breast and prostate cancers.

2. If the leukocyte infiltration is important, you may see clusters or lobules that are close to blood vessels that may be more prone to development of cancer if leukocytes may not reach all tissue cells in the cluster. Is it true?

3. The increasing expression and conversion to nuclear P53 positivity as you go into the core of the clusters revealed in different tissue sections is quite interesting. This suggests that tissue environment which can be altered by hypoxia and leukocyte infiltration and inflammation can influence the outcome of the clusters and cancer development. These changes in tissue microenvironment create a spectrum of cell populations that range from little to high expression of cancer related features. This is an interesting observation and confirms our previous Yin-Yang model of cancer cells (Zhou J, Zhang Y. Cell Cycle. 15;7(10):1360-1370, 2008), where it shows a spectrum of heterogeneous cells in continuum with varying degree of cancer associated gene expression changes and stemness. So not all cancer cells can cause cancer, depending the epigenetic changes, so while different cells have the potentiality to develop into cancer they do not do so if there are no appropriate stress stimuli that cause epigenetic changes. But some cancer cells can develop into "overt" obvious cancer phenotype due to epigenetic changes which determine invasiveness and metastasis that are characteristic of cancer. So in a way, cancer is due to both genetic and perhaps more importantly epigenetic changes (a point we made in the Cell Cycle article attached). You may want to mention this in the Discussion.

4. I think you should emphasize in the Discussion the importance of spatio-temperal continuity of examination of relevant tissues, i.e., clusters or lobules, where your study only examined spatio aspect, which is very important feature of your study. This study touches upon the critical issue of tissue mciroenvironment that plays a vital role in cancer development, which cannot be replicated in vitro cell cultures and partially explain why

despite the numerous studies on cancer relatively little progress has been made and cancer still remains a major unsolved problem based on cell line studies or cell line selected drugs or even target based drugs.

5. If leukocyte infiltration is important for cancer development or spread, anti-inflammatory agents should prevent or reduce the tumor invasion or progression, which is an interesting hypothesis that can be tested. In fact, it is known that aspirin can reduce cancer occurrence and chronic inflammation is associated with carcinogenesis. You may want to mention this (to test the hypothesis) in the Discussion.

6. It seems to me that there are two types of clusters or lobules that are associated with cancer. The first is the one you describe here that is associated in proximity to *in situ* cancer, and the other is the one that is not associated with *in situ* cancer like the one in children but can develop subsequently to cancer. I think we should make it clear here.

7. I think you can end with a new hypothesis of cancer where histological tissues such as clusters or lobules prone to cancer serve as the initial site for subsequent cancer development, upon repeated stresses radiation, chemical, oxidative stresses caused by inflammation due to leukocyte infiltration. In addition, you may propose to further study the significance of such clusters or lobules in cancer by listing a number of things to do, including examining further adjacent cancer tissues, determining the epigenetic changes in different continuous tissue sections spatio-sectioning and association with cancer, propose new approach to cancer prevention and treatment by altering adverse tissue microenvironment including use of anti-inflammatory agents and anti-oxidant and removal of stress.

Reference

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Accumulation of molecular alterations in cells eventually leads to significant morphological changes during the malignant transformation, specific and non-specific? - Challenges for future early tumor detection by molecular biology technology

Mechanisms for the malignant transformation, local invasion and metastasis are very complex. A variety of molecules have been involved in these processes. The article " Malignant transformation and stromal invasion from normal or hyperplastic cells: true or false?" by Dr. Man et al. presented one of most intriguing issues in cancer research. Since the modern medicine started several centuries ago, the diagnosis of malignancies has heavily relied on the morphology of suspected cells and/or tissues. It is already well-known that molecular changes are preceded morphological changes. Our previous studies indicated that the infection by certain species of mycoplasmas could lead to malignant transformation *in vitro* (1). The transformation follows alterations of many molecules. However, during the early stage of the malignant transformation, despite of alterations at the molecular level, many transforming cells remain normal or close to normal morphology (2). Although we could predict time line from the initial changes at the molecular level to obvious changes at the morphological level *in vitr,* it is a huge challenge for us to foresee the similar time line at a clinical setting.

Many researchers believe that it takes 10-16 years for a cancer-initiating cell to develop into a 10-mm^3, clinically detectable tumor. This conclusion is based on some observations that the averaged volume doubling time in most tumors is about 100-210 days during the exponential growth. It might not be true for many types of malignancies. 1) Several kinds of cancers are found in children younger than 10 years old. 2) Clinically, some cancers, such as hepatoma, only take a couple of years or less to develop from a microscopic size to full-below mass. 3) The majority of established cancer cell lines have their doubling times less than 5 days. 4) More than

80% patients with chromosomal alterations detected by UroVysion FISH assay, but no obvious malignant morphological changes will develop clinically detectable bladder cancers within 3-5 years (3). Therefore, we should not be too pessimistic on the issue of early detection of malignancies prior to morphological changes. We should be able to predict tumor development in future by advanced new molecular biology technology. However, even if we do have effective techniques to detect molecular alterations which eventually lead to morphological changes of malignancies, we will still face many challenges. 1) One of biggest challenges is the heterogeneity of malignant cells and tissue architectures as Dr. Man et al pointed out in their paper. The morphological heterogeneity basically reflects molecular heterogeneity among different cell populations in the same area or different areas of tumor. Similar heterogeneity is also present in morphologically apparently normal or benign cells or tissues. Most molecular biology assays require very small amount of samples. Improper sampling could result in a false negative diagnosis. 2) Lack of specific molecular marker(s) for cancer early diagnosis. Although some molecular markers are relatively specific for certain types of cancers, such as fucosylated AFP for hepatoma and some embryo carcinomas, the majority of tumor markers currently used in clinical molecular diagnosis are not very specific, which could be a big problem when they are used for the early tumor detection in morphologically apparently "benign" cells. 3) Short of clear time line from molecular changes to morphological alterations and to normal functional disruption for each type of malignancy, which is the key to predict the occurrence of morphology-changed malignant tumor. 4) Require new techniques which are multiplex, convenient and affordable with short turn-around time. Therefore, we have a long way to predict a malignancy at the molecular level. This will be our future of cancer diagnosis.

Reference

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I enjoyed reading the article you forwarded with its accompanying illustrations. Below is some information related to the article of yours to be published.

Mechanisms and primary molecules involved in tumor invasion and metastasis

1. Migration and invasion are critical parameters in dissemination of cancer cells. If the cells have not developed the potential for invasion and metastasis, appearance in the circulation does not correlate with a later appearance of a metastatic focus. When a D&C is performed in stage I endometrial cancer the survival is 100% after simple hysterectory and yet free tumor cells are frequently detected in the circulation, none of which ever implant to appear as metastatic foci.

2. Metastasis is a major cause of death in cancer patients, which may be due to release of agents such as cachexin or TNF as seen in pancreatic cancer. Migratory cancer cells undergo molecular and cellular changes by remodeling cell matrix adhesion and actin cytoskeleton.

3. In most cells, the loss of E-cahherin (cell surface adhesion molecule) and the gain of mesenchymal markers and premigratory signals occur – epithelial to mesenchymal transition (EMT)

4. Cells become capable of evading immune surveillance. Shedding of mutated mitochondrial DNA into the serum is one major factor paralyzing the immune system so that patients are annergic to tumor antigen.

5. Loss of E-cadherin adhesion occurs in almost cases of malignant progression with an increase in N-cadherin (mesenchymal switch).

6. E-cadherin is one of the transmembrane glycoproteins that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of cancer.

7. A number of growth factors provoke the loss of E-cadherin. This includes TGF-B, hepatocyte growth factor (HGF), members of the EGF family, NOTCH signaling, and Insulin Growth Factor (IGF)

8. The Notch Signaling Pathway (NSP) is a highly conserved pathway for cell-cell communication. NSP is involved in the regulation of cellular differentiation, proliferation, and specification.

9. Hypoxia in the microenviroment of tumors tends to induce receptors for the growth factors for the loss of E-cadherin. Hypoxia is necessary for many forms of metastasis

10. Expression of matrix metalloproteinases (MMP) helps to allow invasion. MMP drugs have been tried to inhibit metastasis.

11. Loss of E-cadherin function leads to disruption and loss of cell polarity liberating cytoplasmic proteins.

12. The juxtamembrane domain of E-cadherin binds to p120 catenin which controls the membrane localization of E-cadherin. The p120 catenin binds to accumulate in the cytoplasm where it represses the activity of RhoA and activates Rac and Cd42. These molecules are GTPases which regulate actin assembly. p120 catenin (p120) is the prototypic member of a growing subfamily of Armadillo-domain proteins found at cell-cell junctions and in nuclei. In contrast to the functions of the classical catenins (alpha-catenin, beta-catenin, and gamma-catenin/plakoglobin), which have been studied extensively, the first clues to p120's biological function have only recently emerged, and its role remains controversial.

13. During the cadherin switch when N-cadherin binds to β-catenin there is a physical linkage to PDGF and fibroblast growth factor and an association with implants to sites of hematoma.

14. Single cell migration has been distinguished into:
a) Mesenchymal migration associated with spindle shaped fibroblast like cells driven by a leading edge with Rac induced cell protrusions.
b) Ameboid cell migration, where rounded cells such as hematopoietic stem cells use a push and squeeze type of migration to make their way thru the extracellular matrix. The movement is driven by RhoA/Rac mediated bleb like protrusions with active myosin/actin contractions

15. Additional migration patterns occur where the cells which maintain their cell-cell junction spread in sheets, strands, tubes and clusters. Here the guiding cells use B1 integrin mediated focal adhesion and local release of MMPs at their leading edges to cleave collagen fibers and orient them in a way that generates tube like micro tracks into which the collective mass of cells migrate.

16. Specific membrane glycoproteins are expressed as mutations that occur in the primary lesion. Each subclone appears to define which site the cell can implant and grow in. Those cells that grow in the liver appear to require a substrate like liver ferritin and as such can only grow in the liver parenchyma. Such cells will not spread to sites such as lung or bone allowing for surgical removal of the metastatic lesion when possible. The reservoir that collects tumors cells is the marrow. Cells can migrate 20+ years later I.e. squamous to scalp and colon flank mass secondary to trauma.

17. One of the surface glycoproteins seen in tumors is podoplanin, a 38kd typre 1 sial mucin. When present it appears to define invasion patterns.

18. When those mutations necessary to allow characteristics of malignancy to appear they probably need a secondary type of process such as transfection with EBV virus to occur. It is known that the transcriptional

protein FGARAT is also associated with overexpression of telomerase. A homologous sequence to FGARAT is found in the EBV virus which probably effects telomerase in a similar fashion.

19. As tumors do develop they do express oncofetal proteins such as the MUC5acs, which help in the development of the GI tract in terms of assuring mucin production. When the process is completed the gene is remethylated so that past pointing doesn't occur. Later in adult life, a mutation occurs in the MUC5ac gene resulting in the expression of a tumor associated immunogenic protein (TAA) which the host recognizes in an inefficient manor to help with tumor surveillance but not complete control. Monoclonals to such a TAA can act as therapeutic agents.

20. The appearance of such TAAs have be shown to appear in normal appearing cell, many months before the phenotypic appearance of malignancy can be defined. Their presence can allow for early treatment, such as cryosurgery of the cervix when ASCUS 1 patterns of pre-malignancy cannot be assured. In the colon and pancreatic tissues, the presence of expressed antigen in normally appearing colonocytes or intraepithelial changes in the pancreatic duct can aid in initiation of early therapy to obviate clinical cancer from developing.

The concept of lymphocyte invasion in many specimens seen on biopsy ie colon and breast cancer and defined as a beneficial response has been shown to be incorrect. At one time the presence of lymphocytes in medullary Ca of the breast meant better response clinically; it is now recognized that the greater the lymphocyte infiltrate the poorer the clinical response. When TIL (tumor infiltrating lymphocytes) cells were cultured and given to patients, no beneficial clinical response was seen. Illustrations by Dr. Man showing lymphocytes carrying tumor cells through membrane to result in metastasis probably explains the poorer prognosis seen with the presence of lymphocytes in the neoplasm.

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I think your observation is significant in questioning current opinion about cancer initiation and progression. It opens many possible projects in cancer research. Obviously, it is very challenging to test some hypotheses that may or may not support your hypothesis. First, whether the nests of abnormal cells are cancer stem cells with invasive features should be tested using xenograft animal model. To do so, it is critical to harvest the individual nests of viable cells from fresh tissue. Second, whether the leukocyte infiltration promotes tumor cell invasion should also be test using xenograft animal model as well as fusion cells. Assuming that the nests of abnormal viable cells freshly harvested would not grow any invasive tumor in xenograft animal model, then, you can test whether the combined mixture or fusion of these cells with leukocytes would. In any way, your observation is important to cancer research and is worthy further investigation.

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This is a very interesting paper that would explain a unique set of cancers that could not be explained by the traditional multi-stage cancer development and progression theory. It could have a profound impact not only in cancer research community but also in cancer management in clinic. Yet, it clearly requires extensive molecular research including epigenetic alterations, non-coding RNA changes, functional studies, etc. to prove it.

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The mechanism of the initiation, transformation, progression and metastasis of cancer has always been regarded as the fundament of guiding and leading to effective therapeutic strategies in clinic practice and research. In this article, based on their careful observations and previous studies that showed a vivid view of hardly detectable changes in the course of malignant tumors in five samples of breast cancer, Dr. Man and his research team raised a question whether malignant transformation and stromal invasion could be originated from morphologically normal or hyperplastic tissues and whether leukocytes could facilitate tumor cell invasion or metastasis. It is obvious that this is a novel hypothesis in the current cancer research field of cancers though it might face more challenges and is to be further verified in more subjects, more effective approaches and even in animal models of breast cancer. This hypothesis would open our paths to seek unrevealed mechanisms of carcinogenesis and metastasis and finally might lead to revolutional therapeutic strategies for preventing and treating human cancers in the near future.