Functional Analysis of PHLDA3, a Target Gene for the Tumor Suppressor Gene p53, and the Contribution of the AMPure XP DNA Cleanup Process

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We interviewed Dr. Rieko Ohki of the National Cancer Center Research Institute, who works on p53, its downstream factors and its role in tumorigenesis. She identified PHLDA, located downstream of p53, as a tumor suppressor gene. Here she discusses with Jun Onodera her work and the importance of the DNA cleanup process in the analysis of mutations in PHLDA3 genes in cancer tissues she is currently analyzing.

[Interviewer: Beckman Coulter Life Sciences, Senior Applications Scientist, Jun Onodera]

What is your research on the tumor suppressor gene p53 and its target factors?

I have been studying the tumor suppressor gene p53 for many years. It is no exaggeration to say that p53 is the most important gene in cancer, as it is known that half of all cancers contain a mutated p53. p53

responds to a variety of stresses on the cell and its job is to ensure that these cells do not become cancerous. In addition, in cases where p53 cannot adequately counter these stresses, it can instruct the cells to die by apoptosis. p53-deficient mice are extremely cancer-prone with 75% dying within a half a year, underlying the significance of p53 in cancer. p53 is a transcription factor that activates the transcription of various genes depending on the intensity and type of stress to which the cells are subjected. p53 halts the cell cycle and prevents overgrowth, and as mentioned above can also trigger apoptosis in some cases. Advances in technology, such as gene expression analysis using microarrays and analysis of the DNA sites to which p53 protein binds in the cell, have enabled a comprehensive analysis of p53 and the identification of now 235 genes regulated by p53. p53 is very well-known and accordingly there are many researchers around the world who have performed analyses similar to ours. We therefore focused our functional analyses on genes whose function were unknown at the time, and this has helped us become one of the world leaders in research on the regulation of cancer by p53.

What can you tell us about the newly discovered tumor suppressor gene PHLDA3?

PHLDA3 is a one of a number of genes of unknown function found through an exhaustive search for potential p53 target genes. Although p53 is designated as a cancer suppressor gene, there are also many other genes that promote cancer, called oncogenes. We do not necessarily want tumor suppressor genes such as p53 to be dominant, and indeed excessive cancer suppression can be harmful to the human body, as this can inhibit cell growth unnecessarily and possibly induce apoptosis. In other words, a healthy cellular state is one in which the functions of oncogenes and tumor suppressor genes are balanced by a kind of mutual antagonism. We have shown that the PHLDA3 protein, which is induced by p53, regulates oncogenic signals by inhibiting the cancer-promoting function of the protein product of the Akt oncogene. When p53 is mutated, PHLDA3 is not expressed, and therefore Akt is not suppressed. As we mentioned earlier, half of all cancers have a mutation in p53, and among the half in which p53 is not mutated, some have been found to lack PHLDA3 function.

This discovery was a key to understanding pancreatic cancer, a disease that caused the death of the Apple founder Steve Jobs. The pancreatic islets of Langerhans secrete hormones such as insulin. When these islets become cancerous we often observe a loss of PHLDA3 gene function and dominance by AKT, a phenotype that indicates poor prognosis for the patients (Figure 1). PHLDA3-deficient mice exhibit abnormal proliferation of the islets of Langerhans, although this alone does not result in cancer in these animals. The relationship between the loss of PHLDA3 function and the promotion of cancer is found not only in the pancreas, but also in tissues such as lungs and large intestine, which are also endocrine tissues. Thus, PHLDA3 may be a universal tumor suppressor gene for endocrine tissues (Ref. 2).

What is your further research progress and how does DNA cleanup affect your results?

Currently, genomic DNA is extracted from cancerous endocrine tissues of the pituitary gland, thyroid gland, and large intestine. These tissue samples are provided by the physician but they can arrive in a variety of forms. Good quality DNA can be extracted if the
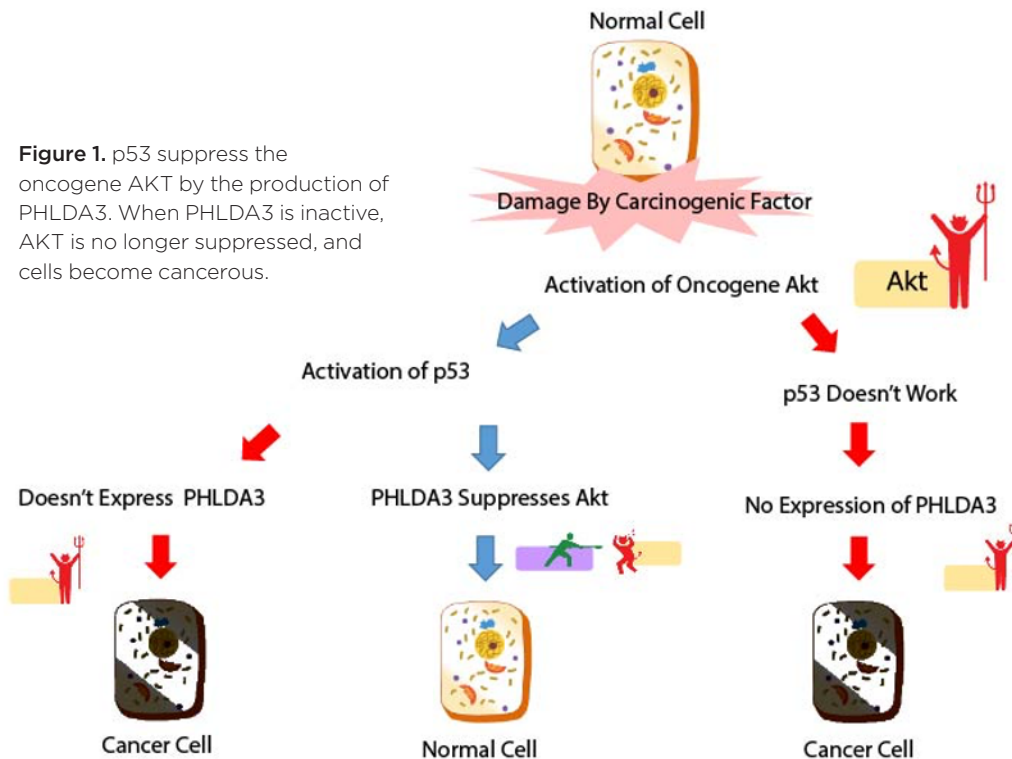

Figure 1. p53 suppress the oncogene AKT by the production of PHLDA3. When PHLDA3 is inactive, AKT is no longer suppressed, and cells become cancerous.

tissue is cut and then immersed in a reagent that stabilizes the DNA. Most tissues arrive as formalin-fixed paraffin-embedded (FFPE) specimens and some form of DNA fragmentation is inevitable in certain specimens due to the effects of formaldehyde. Even more troublesome is dealing with small sample sizes, such as microdissected specimens of the FFPE tissue, which involves cutting and collecting the target cell mass from a piece of tissue under the microscope.

Here we show the results of microdissection of a sample from an FFPE tissue section. The sample was deparaffinized with xylene, which also functions to lyse the cell and de-crosslink the contents. DNA was then extracted by the phenol/chloroform method. However, as shown below the DNA extracted did not amplify well. As the number of PCR cycles increased an amplified band appears, but the same band also appears in the negative control, which makes it impossible to proceed with a PHLDA3 mutation analysis (Figure 2A). We therefore used AMPure XP, Beckman Coulter's magnetic bead-based DNA cleanup reagent kit, to further purify the DNA and succeeded in obtaining an amplified band of the correct target band size that was not seen in the negative control (Figure 2B). DNA extracted by the phenol/chloroform method contains many short DNA fragments and low molecular-weight compounds, which may interfere with the PCR reaction. AMPure XP seems to be able to efficiently remove short DNA fragments of 100 bp or less, as well as salts.
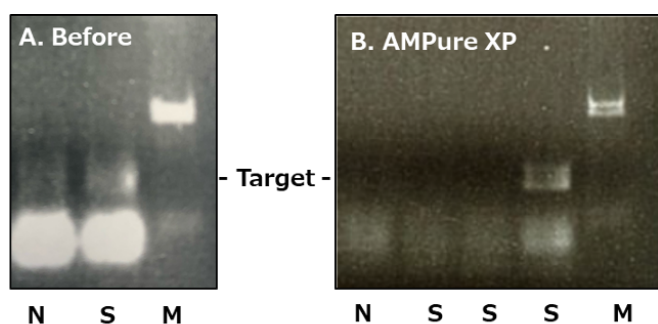

Figure 2. DNA purified with AMPure XP detects specific amplified bands not found in negative controls N: negative controls, S: Sample, M: size marker.

Did you use magnetic bead purification in your Sanger sequencing analysis?

The amplified PCR fragments were sequenced by Sanger sequencing to analyze potential PHLDA3 mutations. Before performing Sanger sequencing on the PCR amplification product, it is generally necessary to have a purification step to remove primers and unreacted dNTPs. Previously, we used a silica column-based purification kit, but we saw background in the resulting sequence chromatogram (arrow in Figure 3A). Although not a problem if one is simply analyzing the sequence, this background can significantly interfere with the detection of low-frequency mutations found only in some cells. Therefore, we switched from the silica column kit to AMPure XP. After purification and sequencing, primers and short DNA fragments that could not be removed with the silica column kit appear to have been removed with AMPure XP, and the background was clearly suppressed (arrow in Figure 3B).
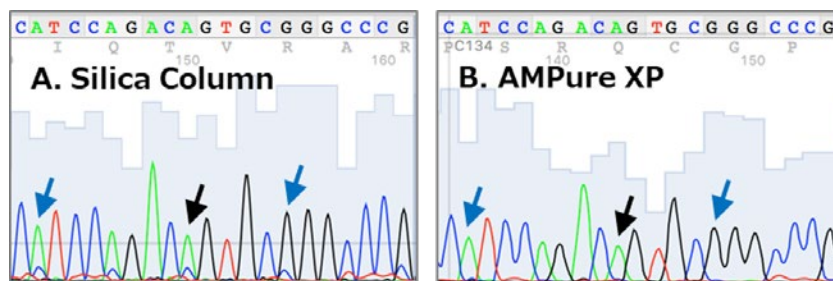

Figure 3. AMPure XP purification of PCR amplification products reduces sequencing chromatogram background

In addition, following the Sanger sequencing reaction, we used the CleanSEQ cleanup reagent kit to remove primers and unreacted fluorescent dyes (ddNTPs) before loading the sample on the sequencer. Thus, each of these processes is based on the use of magnetic beads. Among Beckman Coulter's magnetic bead products, there is also FormaPure XL, which is a nucleic acid extraction kit designed for FFPE tissue. Therefore, one can standardize the entire workflow using magnetic bead kits for DNA extraction and purification starting from FFPE tissue through to sequencing.

We look forward to participating in p53 research!

The tumor suppressor gene, p53, is recognized by researchers in the field as one of the most important genes in cancer research. While it may seem that p53 has already been well-studied by many researchers, and that many of its functions have already been elucidated, we are pursuing our research with the conviction that there remain many functions yet to be discovered. We also believe that p53 will be an important target in future applications such as cancer treatment and diagnosis. In contrast to receptors and transporters located on the cell surface, p53 is a transcription factor located in the nucleus and has thus been regarded as a difficult drug target. However, inhibitors of nuclear factors have been more successfully developed in recent years, and this makes modulation of p53 of increasing importance in both basic and applied research. Younger researchers may imagine that most cancer genes are already known and understood, but I remain interested in p53 as an interesting and important factor, and sincerely hope that they will participate in this research if possible.

A good opportunity to find out about the latest p53 research is the 10th International MDM2 Workshop hosted by me in September 2022. While this conference is nominally about MDM2, a well-known oncogene that suppresses p53 function, there will be many presentations on p53, and more than 150 top-level p53 researchers from around the world. We are anticipating the active participation of Japanese cancer researchers, especially young researchers, and look forward to hearing the latest basic and applied discoveries in this promising area of research.

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