4-3-2017

The Optimization of the Nuclear Protein in Testis (NUT) Antibody and its Importance and Impact in the Pathology Lab

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The Optimization of the Nuclear Protein in Testis (NUT) Antibody

and its Importance and Impact in the Pathology Lab

by

Lindsey Martinez

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
Department of Environmental and Occupational Health
College of Public Health
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Date of Approval:
March 29, 2017

Keywords: Nuclear Protein in Testis, NUT Midline Carcinoma, immunohistochemistry, optimization, pathology, glandular differentiation

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Acknowledgments

I would like to thank the histology team at Moffitt Cancer Center and the opportunity they provided for me. The immunohistochemistry professionals, Jodi Balassi, HTL, Carmen Rivera, HTL, and Quiana Tolbert, HTL made the technical part of this process come to fruition. I would also like to thank the leadership provided by Farrah Kahlil, M.D., Helen Molina, Lab Manager and Malissa Snyder, Supervisor. Finally, none of this would have been possible without the guidance from Giffe Johnson, Ph.D. and the other professors who are part of the toxicology program at the University of South Florida.
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Abstract

Optimization in immunohistochemistry is often a time consuming and complex process. There are a varying array of moving parts to consider all while preserving the sensitivity and specificity of the test. When optimizing an antibody it is important to consider the fixation of the tissue and the type of epitope retrieval that would be best suited for the test. The dilution of the primary antibody is a key marker for the efficiency and effectiveness of the laboratory protocol. The purpose of this study was to produce an optimized antibody for the nuclear protein in testis to detect NUT midline carcinoma that provides a sensitive and specific test but is also efficient and can be useful for everyday pathological dedications.

The midline carcinoma defined by the translocation of the NUT gene on chromosome 15q14 that bonds with BRD4 or BRD3 commonly known as NUT midline carcinoma (NMC) is a rapidly aggressive and fatal disease. Commonly a fluorescent in situ hybridization (FISH) test is used to diagnosis this carcinoma. This test takes longer than traditional IHC and can delay the treatment of the patient. Therefore this is why, irrespective of the levels of tumor markers, immunohistochemistry for the NUT marker should be performed in any case where there is poorly differentiated carcinomas that do not have glandular differentiation that come from midline structures.
Introduction

To begin, we must start with a basic background on immunohistochemistry. Techniques involved with immunodiagnostics have been around for more than 60 years but only in the last 20 have they seen progression [1]. This is due to the unpredictable quality of polyclonal antibodies. There has been substantial advances in immunohistochemistry and this has had a dramatic impact on laboratory medicine. In most laboratories, including the one this project was completed in, immunohistochemistry has become as routine and sometimes more relied upon than any other special stain. First, a refresher on some general immunology, focusing mainly on the humoral aspect. An antigen is a substance that can induce a demonstrable immune response. The most common of these antigens that tend to produce antibody production are viruses and bacteria. Immunoglobulins, or more commonly known antibodies, are proteins that are produced by B lymphocytes in response to an antigens stimulation. There are 5 major classes of antibodies: Immunoglobulin (Ig) G, A, M, D, and E. Each one of these are different in their respective function and structure. While, each class is different there is an overall basic “Y” shape to the antibody molecule, with the upper arms binding to the antigen. Within this structure there are two identical heavy chains (gamma, alpha, mu, delta, or epsilon) and two identical light chains (kappa or lambda). The binding regions for antibodies on the antigen are referred to as epitopes. In regard to how antibodies are produced, when a lymphocyte is exposed to an antigen proliferation can occur. When this transpires each
lymphocyte can form a clone cell. These clones produce an identical antibody. Polyclonal antibodies are formed when various clones produce antibodies of different classes and these form a pool. Generally, antibody production is made from an injection into an animal. An isolated immunoglobulin antibody is injected into usually a mouse or rabbit, but all different kinds of animals have been used. This injection stimulates the antihuman immunoglobulin antibody reagent. There is high variability in the immune response from one animal to another therefore polyclonal antibodies can be difficult to standardize. The development of monoclonal antibodies changed the way immunohistochemistry was used. Monoclonal antibodies are developed by injecting an animal, for example a mouse, with an antigen. The B lymphocytes that are produced by this mouse are then harvested and the cells are fused with nonsecreting myeloma cells. This yields a hybrid cell that keeps the antibody secreting qualities of the lymphocyte and that allows an unending production created by the tumor cells. This advance provides a reduction in non-specific antibodies, higher homogeneity, and no variability in lot to lot or batch to batch fabrication. Monoclonal antibodies produce tests with higher affinity and sensitivity, this also serves to reduce background staining.

There are various methods that can be used in immunohistochemical staining. There are four methods that will be outlined for the purposes of this paper; direct, indirect, unlabeled or soluble enzyme immune complex, and avidin-biotin. The direct method uses a labeled antibody of known specificity to identify antigens in the patient tissue [1]. The indirect method uses the patient’s serum and is added to tissue sections containing the desired antigen, this tests to see if the antibodies are present in the patient to those antigens. These two techniques (direct and indirect) usually involve immunofluorescence. The unlabeled or soluble enzyme
immune complex method is a three step process. A primary antibody, a linker or secondary antibody and a soluble enzyme-antienzyme complex are used. This method is based on the peroxidase-antiperoxidase (PAP) technique. The final method discussed is the avidin biotin. There are two ways of completing this technique: the avidin-biotin complex or the labeled avidin-biotin. This is achieved through the high affinity that avidin has for the vitamin biotin. This binding is considered irreversible which can be very desirable to the pathologist. In both methods the primary antibody is followed by a biotinylated secondary antibody. This is then followed by the complex or the labeled avidin-biotin. The complete methods for the antibody of topic will be discussed further.

Optimizing antibody expression can be difficult and time consuming. It is a varying array of different interactions and components that need to be carefully managed to provide the pathologist with the ideal results. Immunoreactivity can be compromised by the use of certain fixatives, in particular aldehyde fixation [1]. Though, in the majority of laboratories today we use formalin fixations, alternatives do occur for various reason. Over fixation with formalin has its own issues as well. It may cause excessive crosslinking of the proteins in the tissue. This can hinder the antibodies ability to access the epitopes they need. The major problem with this is that false-negative stains may result. The issue caused by crosslinking can be resolved by the use of epitope enhancement/antigen retrieval. There are several benefits to using this enhancement, like the capability to dilute antibodies and open exposure to epitope sites that were not previously available. This retrieval can also lead to more uniform staining, a quicker reaction time, and decreased background staining. There are two methods for antigen retrieval: heat induced epitope retrieval (HEIR) and enzyme induced epitope retrieval (EIER). To begin
with the heat induction, formalin fixed tissue is immersed in a metallic salt solution and heated
(usually in a microwave system but this can be done in a pressure cooker system, steamer and
autoclave system) to 100 degrees Celsius. One of the most widely used solutions is a sodium
citrate buffer (0.01 M, pH 6.0). A recommended solution has a high pH for surgical pathology,
like a TRIS-HCl or sodium acetate. Technicians need to be careful because there are also
disadvantages to using a high pH because these can damage the tissue sections. Some of the
HEIR methods can cause loss of tissue, burns, morphological damage, and the possible
destruction of antigenicity. It is important to note safety precautions as well since some of the
solutions, especially when heated are toxic, so a hood and proper personal protective gear
should be worn. Each antibody is different and therefore they should be evaluated with the
protocol of no retrieval, retrieval with just heat, retrieval with just enzyme and with the
combination of the two. These steps should yield the optimal solution. The next method to
discuss for epitope enhancement is enzyme induced epitope retrieval (EIER). Proteolytic
enzyme digestion is the older of the two methods of epitope retrieval. The most common
enzyme used for this process is trypsin. Generally, the trypsin should contain 0.1% calcium
chloride or a combination of a 0.1% trypsin solution in phosphate buffered saline. A 10 minute
digestion in the trypsin solution at 37 degrees Celsius will unmask most antigens, however,
cytokeratin’s usually require longer digestion times between 30 to 60 minutes. There is no
universal proteolytic agent. Therefore various enzymes solutions can be used, some
documented ones include: 0.1% protease in phosphate buffered saline (common in FISH
testing), 0.1% pronase in 0.5M TRIS buffer, 0.6% fican, and 0.01N hydrochloric acid. Proteolytic
enzyme digestion can reduce nonspecific staining but it can also increase it if not properly used.
It is also a possibility that it may create false-negatives by weakening specific staining. A basic guideline for developing protocols for new antibodies begins with no retrieval. It then advances to heat retrieval, then enzyme retrieval and finally a combination of the heat and enzyme inducement. The results are then compared. In the case of the nuclear protein in testis antibody, articles have been published on successful protocols that have been previously tested so these provided a base to build up from. Moving on to the next important section of optimization, choosing the correct controls and addressing quality control issues. Positive controls must be run alongside each antibody when immunohistochemistry is performed. In regard to the NUT antibody, the nuclear protein in testis is confined normally to the germ cells in the testis and ovary [6]. Therefore, for the purposes of optimization of the NUT antibody, a testicle was used as the control. Fixation and tissue preparation can vary lab to lab and it is important to have a working control that was prepared in the same conditions as the testing tissue [1]. In some cases, it is appropriate to run a negative control as well. This can be done by substituting the primary antibody with a nonimmune serum from the same species as the primary antibody or using a diluent as a replacement for the primary antibody. However, if the diluent is used the negative control will not be able to define nonspecific binding of animal serum to the tissue. The laboratory that this antibody was optimized and validated in follows the College of American Pathologists guidelines for immunohistochemistry. Optimal dilution is also another factor to consider when bringing a new antibody into a working status, this can be seen in our methods under the titer. This affects the specificity and sensitivity of the stain. Generally, a manufacturer will have an approximate dilution for usage. Cell Signaling, the
manufacturer of the NUT antibody, suggested a 1:45 starting point. A basic formula can be seen in figure 1 below. A base of 1000 µL is used.

\[
\frac{1000}{x} = \mu\text{L of raw antisera}
\]

\[
1000 - \frac{1000}{x} = \mu\text{L of antibody dilution buffer}
\]

**Figure 1** Example of equation for dilution

If an undiluted antibody is the form that the product comes in, which was the case for the NUT, it is best to dilute only the amount needed for the current run in use. The storage of antibodies are also an important factor. The nuclear protein in testis antibody from Cell Signaling comes in a frozen non-dilute form and this is the way it is stored [2]. The antibody was supplied in a 50% glycerol and less than 0.02% sodium azide, 10mM HEPES (pH 7.5), 100µg/ml BSA and 150mM NaCl. It is recommended to be stored at -20 degrees Celsius and not aliquoted. The freezer that the antibody resides in is checked on a daily basis in accordance to CAP guidelines. Within the protocol for immunohistochemistry, it is important to discuss the use of blocking reactions.

There are two methods that are part of the immunoperoxidase methods [1]. The use of hydrogen peroxide in a solution of absolute methanol is used to block endogenous peroxidase activity. This blocking reaction is crucial if the tested tissue contains numerous red blood cells. The second type of blocking is needed when nonspecific background staining occurs from the
result of an antibody attachment to charged connective tissue or collagen. If the first solution applied to the tissue is the primary antibody, nonspecific staining can occur. Thereafter, the secondary antibody will bind to this nonspecific antibody and when stained with the chromogen it will result in positive nonspecific staining. This can be prevented by adding an innocuous protein solution to the tissue prior to the application of the primary antibody. This protein will adhere to the charged sites and avert the nonspecific binding. The innocuous protein solution is generally made from the same animal species that the secondary antibody is. This serum can be applied just before the primary antibody with an incubation of 10 to 20 minutes. The final discussion point in the immunohistochemistry protocol is the DAB reaction. DAB is a brown chromogen most commonly used in immunohistochemistry. There are other options available but DAB is the most stable, reliable and visually appeasing when performing routine IHC. As far as optimization standards there are several methods that can be used to intensify the DAB chromogen. Heavy metals can be used by supplementing or used after the incubation. Some common solutions are 1% nickel chloride, 1% cobalt chloride, or 1% copper chloride. Using this method there is an increase risk in creating background staining. This can mask and make the detection of the true antibody difficult. Also, some metals should not be used with nuclear antigens especially if a low hematoxylin counterstain is used. Another method for DAB intensification is the use of imidazole. Adding in 0.01M imidazole to the chromogen incubation at a pH of 7.6 will help increase the sensitivity and the efficiency of the detection of the DAB. It is important to note that imidazole hinders the pseudoperoxidase activity of hemoglobin. Finally, the use of osmium tetroxide can also aid in the intensity of the DAB reaction. This product is always used after the DAB reaction takes place. The DAB reaction
product is osmiophilic and thus will continue to darken with the use of post staining osmification. This reaction will also assist in the prevention of fading of the final stain if the sample is to be stored. As with other DAB intensification reagents, there is the possibility of increasing the staining of the background and intensifying the reaction product. The use of osmium tetroxide boosts the staining intensity but does not increase the efficiency of the detection.

**Objective:** The purpose of this study was to produce an optimized antibody for the nuclear protein in testis to detect NUT midline carcinoma that provides a sensitive and specific test but is also efficient and can be useful for everyday pathological dedications.

**Hypothesis:** A hypothesis that the NUT antibody would be optimized at a 1:100 dilution using a standard protocol on an automated immunohistochemistry platform.

**Research Questions:**

1. What is a dilution of the NUT antibody (rabbit monoclonal) that would be sensitive and specific?
2. What is the optimal protocol for the NUT antibody for efficient use in a pathology laboratory?
Methods

There are several categories to consider when optimizing an antibody for immunohistochemistry. First research needs to be done on the manufacturer (if there is more than one), the type of clone used, the ease of usage, cost, consistency and reliability of supply. After looking up several vendors, the manufacturer Cell Signaling was picked, mainly because it was a C52 monoclonal rabbit antibody [2]. Cell Signaling carries Rabbit mAb an IgG isotype (C52B1). It detects endogenous levels of total NUT protein. This antibody can also detect endogenous levels of BRD4-NUT fusion protein that is found in NUT midline carcinoma. It has a species reactivity to human and rat and a 100% sequence homology in monkeys.

There were numerous steps in the process of determining the proper protocol for this antibody. Over a period of time ten runs were done on different immunohistochemistry platforms at various dilutions, this can be seen clearly in Table 1. The process began on the Dako Omnis platform where a 1:100 basic dilution was made and a standard enzyme and heat combination retrieval was performed. A 20 minute horseradish peroxidase blocking agent was added and the antibody was incubated for 1 hour as recommended, this can be seen in Figure 6. There were five more protocols ran similar to this first one, slowly increasing the blockage and adding in a polymer linker, still with a dilution at 1:100. After little success in being able to create an intense DAB reaction that would create a nuclear stain that was consistent throughout, a 1:50 dilution was tried, this can be seen in Figure 8. After this trial it was decided
to switch to a different platform, the Ventana BenchMark Ultra. The same test was repeated on
the BenchMark. The next step involved repeating the same protocol with a 1:25 dilution. The
final protocol was established on October 28th, 2016 which is discussed in detail below. A final
dilution of 1:25 with amplification was used for the optimized protocol. This progress can be
seen in Figure 8. One more test was ran after the optimization to see if an ultrawash would help
reduce background and nonspecific staining but since the test is a nuclear stain the wash step
was deemed unnecessary. The ultrawash can be seen in Figure 7.

The final optimization and validation was completed on the Ventana BenchMark Ultra
IHC/ISH staining module. There are a total of 109 steps for simplicity and comprehension some
parts have been summarized and combined. The complete procedure is as follows: Enable the
machine, set default temperature to 72 degrees Celsius, deparaffinization of slides occurs over
the next four minutes, rinse steps, then cool for 8 minutes. Long cell conditioner #1 is applied
and the temperature is set to 95 degrees Celsius and incubated for 8 minutes. Next, cell
conditioner #1 is applied 11 times followed by the short cell conditioner #1, this is incubated for
8 minutes. Slides are rinsed with reaction buffer and warmed to 36 degrees. The UV inhibitor is
applied for 4 minutes than rinsed with reaction buffer. The slide is now warmed to 37 degrees
Celsius and prepped for the antibody to be titered. The primary antibody is hand applied and
incubated for 32 minutes. Slides are rinsed with reaction buffer and warmed to 36 degrees
Celsius. One drop of amplifier B is applied and incubated for 8 minutes, slide are rinsed with
reaction buffer. One drop of amplifier A is applied and incubated for 8 minutes, then again
rinsed with reaction buffer. One drop of UV HRP UNIV MULT (horseradish peroxidase) is applied
and incubated for 8 minutes. Slides are rinsed with reaction buffer and one drop of UV DAB and
one drop of UV DAB H2O2 is applied and incubated for 8 minutes and rinsed with reaction buffer once again. One drop of UV COPPER is applied and incubated for 4 minutes, then rinsed with reaction buffer. One drop of hematoxylin (counterstain) is added and incubated for 4 minutes, then rinsed with reaction buffer. Finally one drop of bluing reagent (post counterstain) is applied and incubated for 4 minutes. The slides are rinsed thoroughly with the reaction buffer and the heat disabled. The slides are then prepped for coverslipping and checked for accuracy.
Results

Optimization was achieved with a 1:25 dilution using an amplification protocol. This was achieved over 10 trials with 3 different dilutions and 8 different protocols. The first trial consisted of a 1:100 dilution on the Omnis platform, this test provided inaccurate staining and lacked enough DAB intensity to deem the staining nonspecific by the overseeing pathologist. This trial is seen in figure 7. The second trial was continued on the Omnis and was also done with a 1:100 dilution, this trial also had results that were not sensitive enough, the same as the first. The third trial was also done on the Omnis and the protocol was the same but the horseradish peroxidase blocking reagent was increased 10 minutes, the results again were the same as the first two trials, imprecise and not intense enough. The fourth trial was also done with a 1:100 dilution and a 30 HRP but increased the enzyme retrieval by 10 minutes. This trial increased staining intensity very slightly but would still be considered to be nonspecific staining by the pathologist therefore we continued with our trials. The fifth trial continued with the 1:100 dilution but increased the blocking reagent an additional 10 minutes in the hope it would increase the specificity. Unfortunately, there was no change and still produced inexact staining. With the sixth trial we increased the dilution to a 1:50 concentration, this can be seen in figure 6. The staining can be seen to increase the intensity of the DAB reaction in the nuclear stain but it was not staining evenly throughout the control. Also in figure 6 trials 7, 8, and 10 can be seen. In the seventh trial after a lack of intensity we switched IHC platforms to the Ventana
BenchMark. This trial was also done with a 1:50 dilution with the blocking reagent at 40 minutes. There was a slight increase in concentration of the DAB in the nuclear stain but again was not staining the entire control evenly. In trial eight the dilution was increased to a 1:25 concentration finally this showed an intense increase in the strength of the DAB reaction. The pathologist wanted to ensure encompassing staining so in trial nine the same protocol as eight was ran but with an amplification step added in. This was the protocol that was approved officially by the pathologist due to its intense specific DAB staining that accurately encompassed all of the nuclear detail required throughout the control. Finally in the tenth trial the same protocol as eight and nine was used but an ultrawash step was added in. This was done in order to help reduce nonspecific staining but ended up reducing overall staining so the decision was made to not use the wash. With the approval of the overseeing pathologist the test is designed to be sensitive in both control testicle tissue and the patient tissue and specific to the nuclear protein in testis antibody.
Tables
Table 1. Compilation of the 10 protocols used to optimize the NUT antibody

<table>
<thead>
<tr>
<th>Date of Run</th>
<th>Protocol #</th>
<th>Titer</th>
<th>Cell Conditioning Enzyme Treatment</th>
<th>Amplification/ Ultra Wash</th>
<th>Primary Antibody Incubation</th>
<th>Primary Antibody Temperature</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-27-16</td>
<td>1</td>
<td>1:100</td>
<td>HRP 20</td>
<td>none</td>
<td>60</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td>6-15-16</td>
<td>2</td>
<td>1:100</td>
<td>HRP 20</td>
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<td>60</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td>6-17-16</td>
<td>3</td>
<td>1:100</td>
<td>HRP 30</td>
<td>none</td>
<td>60</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td>8-5-16</td>
<td>4</td>
<td>1:100</td>
<td>HRP 30/ Poly 10</td>
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<td>60</td>
<td>37° C</td>
<td>10 minutes of polymer added</td>
</tr>
<tr>
<td>10-21-16</td>
<td>5</td>
<td>1:100</td>
<td>HRP 40/ Poly 10</td>
<td>none</td>
<td>60</td>
<td>37° C</td>
<td>10 minutes of polymer added</td>
</tr>
<tr>
<td>10-25-16</td>
<td>6</td>
<td>1:50</td>
<td>HRP 40</td>
<td>none</td>
<td>60</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td>10-26-16</td>
<td>7</td>
<td>1:50</td>
<td>HRP 40</td>
<td>none</td>
<td>60</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td>10-27-16</td>
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<td>1:25</td>
<td>HRP 40</td>
<td>none</td>
<td>60</td>
<td>37° C</td>
<td></td>
</tr>
<tr>
<td>10-28-16</td>
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<td>1:25</td>
<td>HRP 40</td>
<td>Amplification</td>
<td>32</td>
<td>37° C</td>
<td>Selected Protocol</td>
</tr>
<tr>
<td>11-1-16</td>
<td>10</td>
<td>1:25</td>
<td>HRP 40</td>
<td>Amplification and Ultra Wash</td>
<td>32</td>
<td>37° C</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 2.** Image of the basic Dako Omnis Immunohistochemistry platform on which the beginning of the optimization protocol experimentation
Figure 3. Images of the Ventana Benchmark Ultra immunohistochemistry platforms where the final optimization protocol was created for NUT antibody
Figure 4. The first two steps in performing a titer, the technologist makes the specific amount of diluent and retracts the liquid into the pipette
Figure 5. The last two steps of the titer, the technologist carefully adds the specific amount of diluent and antibody to the slide covered with buffer, then the tech ensures coverage of the tissue with the antibody.
Figure 6. These 4 images represent the progression of dilution and the altering of the immunohistochemistry protocol. A is a 1:50 dilution, with standard retrieval, performed on the Omnis Dako platform. B is a 1:50 dilution, with standard retrieval, performed on the Ventana BenchMark Ultra platform. C is a 1:25 dilution, with standard retrieval, on the Ventana BenchMark Ultra. D is a 1:25 dilution, with amplification retrieval and an ultra wash, on the Ventana BenchMark Ultra. With each image the increase in the intensity of the DAB chromogen can be seen, the final product displays even staining reaching even the lightest nuclear antibodies but the ultra wash reduced some of the crispness of the DAB.
Figure 7. The first image of a 1:100 dilution, the staining here is very light and could be considered non-differential

Figure 8. This is the final optimized protocol a 1:25 dilution with amplification
Discussion

To understand the importance of the NUT antibody we must first understand the cancer that it diagnosis’. NUT midline carcinoma (NMC) is a genetically defined, aggressive cancer [5]. NMC is very rare, and difficult to properly diagnosis disease. Since it is only newly recognized its true indices are unknown. It is known by midline carcinoma by children and young adults with NUT gene rearrangement, thymic carcinoma, poorly differentiated carcinoma, or as it is known by the World Health Organization carcinoma with t(15;19)translocation [8]. It is so rare that frequency is difficult to determine. In studies, 7% of the 98 patients with carcinoma under the age of 40 were NUT midline carcinomas [5]. In a wider age range study, where the average was 47, 18% of patients with poorly differentiated carcinomas of the upper aerodigestive tract particularly the sinonasal region, were NMC. NUT midline carcinoma is defined by the NUT gene rearrangement. In the majority of cases, coding on chromosome 15q14 is bonded with BRD4 or BRD3. This creates chimeric genes that encode BRD-NUT fusion proteins. In the remainder cases that do not bond with BRD, fusion occurs with an unknown partner gene, these tumors are known as NUT-variant. The diagnosis of NMC can be made by displaying the NUT fusion protein with a monoclonal NUT antibody. In studies it has been shown that NMC can affect people in age ranges from 3 to 78. Since NMC is genetically defined it does not arise from a specific organ. It has been recorded to occur mainly in the upper aerodigestive tract and the mediastinum. There has, however, been cases that have come from the bone, bladder, salivary
glands, pancreas, and the abdominal retroperitoneum. In studies survival is rare. One case, which will be discussed later, has survived out of all of the studies.

In 1999 the first known American case of NUT midline carcinoma developed [5]. A 12 year old girl developed a sore throat and when antibiotics did not take affect the patient was taken to an otolaryngologist where a mass was discovered on her epiglottitis. A biopsy was taken and the tumor resembled a nasopharyngeal carcinoma. She was treated but the cancer was aggressive and rapidly lethal. During her time in the hospital the biopsy was analyzed and her karyotype held a very distinct abnormality, a t(15;19)(q13;p13.1). Dr. Johnathan Fletcher, who studied rare solid tumors with translocation, noticed in his literature review that there was also three other cases with the same translocation. The cases originated in Japan and Dr. Kubonishi had created a cell line from one of the patients. The cell line was used to create gene mapping, using the breakpoint method. Over the period of a year, the cell line was successfully mapped and FISH probes were developed for screening. The most exclusive thing about the NUT midline carcinoma could be its simple karyotyping. There is generally only a single abnormality. In this regard, it would resemble more of a leukemia rather than a carcinoma which is why the fusion oncogene is so vital. The NUT promoter region is only active in adult testis and ciliary ganglion hence only one of the two fusion genes express. The breakpoints occur within the intron 10 of BRD4, intron 9 of BRD3, and 2 intron of NUT. These fuse with BRD4 and encode both the acetyl-histone binding bromodomains and the extraterminal domain with the entire NUT gene. With only one exception, all diagnosed NUT midline carcinoma cases are fatal. The average survival is only 9 and a half months, regardless of aggressive treatment. Some observations suggest that the NUT-variant type of abnormality may live longer than the
BRD4-NUT gene rearrangement. There is a suggested connection of the BRD4 and NUT in chromatin regulation. BRD4-NUT could modify chromatin so that it would prevent the expression of genes that are required for epithelial differentiation. As with acute promyelocytic leukemia, retraction of BRD4-NUT or BRD3-NUT activity in nut midline carcinoma through siRNA-mediated knockdown reveals an induction of squamous differentiation followed by growth arrest. A three week study discovered that the differentiation is irreversible. These findings are important because they could suggest that BRD4-NUT is responsible for maintaining cells in a continuous state of proliferation by blocking differentiation and that BRD4-NUT is potentially a powerful therapeutic agent.

Case Study

The first and only successful treatment from NUT midline carcinoma comes from a 10 year old boy who tested positive for BRD/NUT undifferentiated tumor in the iliac bone [8]. The course of this disease is always invariably fatal regardless with the treatments of chemotherapy and radiotherapy, this case however is different. In 1991, a 10 year old boy presented with weight loss, fever, fatigue and increasing pain in the right hip. X-ray, CT scans, and a MRI exposed a 10cm tumor in the iliac bone that had spread to the soft tissues. After this discovery, a fine needle biopsy was done and displayed what was suggestive of malignant small round cell tumor. A surgical biopsy was taken and revealed a high nuclear to cytoplasmic ratio and neoplastic cells with high mitotic activity. Immunohistochemistry staining was performed with the following tests: chromogranin A, desmin, S100, neuron-specific enolase, and leukocyte common antigen. From these tests it was most likely to be determined small round cell tumor, Ewing Sarcoma. Further cytogenetic revealed the t(15;19)(q13, p13). The patient’s treatment
The plan was selected as combined modality therapy, which is the protocol for inoperable Ewing Sarcoma. The program combined four chemotherapy cycles, consisting of two courses of VAI (vincristine, ifosfamide and doxorubicin) alternating with PAI (cisplatin, ifosfamide and doxorubicin) this occurred at three weekly intervals. The treatment transpired over a period of 35 weeks, following the patient received a cumulative dose of 450mg doxorubicin, 12mg vincristine, 360mg cisplatin, and 56 g of ifosfamide per m2 body area. During the initial first and second cycles hyperfractionated accelerated radiotherapy at 1.5 Gy twice daily to a total dose of 60 Gy was administered. After therapy a surgical biopsy was performed finding no viable tumor cells.
Conclusions

Although NUT midline carcinoma right now is an invariably fatal disease with rapid progression, the opportunity for clinicians to have reliable rapid results with immunohistochemistry from the NUT antibody could provide some extension on the patient’s life span or in one case if caught early and treated aggressively may allow a somewhat normal life. The capability of rapid accurate results from the immunohistochemistry NUT antibody would be able to allow the pathologist to report results sooner than the commonly used FISH technique, which in this particular case of carcinoma is vital. The correct sensitivity and specificity that correlates with the optimization of the nuclear protein in testis antibody provides a comprehensive and reliable test and displays why it is so indispensably important for this laboratory proficiency to be tested properly.
References


