ASSESSING THE ASSOCIATION BETWEEN THE INCREASED RESOLUTION OF THE SIGNATURECHIP WG AND THE ABNORMALITY DETECTION RATE

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Abstract

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Microarray technology is quickly becoming the test of choice in diagnosing genetic disorders of patients with mental retardation, developmental delay, dysmorphic features, and seizure disorders. Signature Genomic Laboratories has developed a new version of the microarray with increased resolution. The SignatureChip, has the ability to detect losses or gains of genetic material as well as the location of abnormalities, many of which correspond to particular diseases or defects that are treatable with proper health care. The purpose of this study is to determine if the increase in resolution, from SignatureChip Version 4.0 to SignatureChip WG, is associated with an increase in the abnormality detection rate.

Two hundred twelve patients were tested using both versions of the microarray. Twenty-four of the 212 patients had abnormal results using SignatureChip Version 4.0 corresponding to an 11.3% abnormality detection rate. As requested by the patients' physicians, these patient samples had follow-up testing with the newer version of the microarray, SignatureChip WG. Of the 212 patients, 56, including the original 24 patients with abnormalities, were found to have abnormal results, resulting in an abnormality detection rate of 26.4%. These results lead to a

McNemar's chi square value of 32.0 with an associated p value of less than 0.001 and one degree of freedom. The difference between the abnormality detection rates of the two microarrays, as denoted by the p value, is statistically significant.

The introduction of microarrays into the clinical setting has proven as effective, if not more effective, in detecting gains or losses in patients' genetic makeup. While this technology continues to show promising advances in genetic diagnostic testing, analysts must pay particular attention to unclear copy number variants. These are common in the normal population and too much reliance on them results in inaccurate reporting.

The results of this study have a number of implications for patients, their clinicians, and Signature Genomic Laboratories. It is clear that the evolution of this technology is inevitable, and this study provides evidence that for now, the evolution has value for the patients and the company in an increased detection rate.

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Dedication

I dedicate this thesis to my husband, Jake, who provided me

with a tremendous amount of support and confidence to accomplish my goals and ambitions.

CHAPTER ONE

INTRODUCTION

This section discusses the purpose of the thesis and states the hypothesis. This section includes a brief review of the literature on the topic of genetic testing with the use of microarrays and the association with resolution. The section concludes with a discussion of the significance of this type of study and the contribution to the current state of knowledge in this field.

Study purpose

The world of health care and genetics has become increasingly intriguing and complex. Because of the advances in technology, scientists are now able to develop new techniques that advance the way patients are diagnosed and treated. One particular advance in the genetic diagnostic world is the use of the microarray with comparative genomic hybridization (CGH) (Pinkel, Segraves, Sudar, Clark, Poole, Kowbel, 1998), which is the technology Signature Genomic Laboratories, LLC in Spokane, Washington has used to diagnose thousands of patients with genetic disorders. This laboratory test, the SignatureChip, has the ability to detect losses or gains of genetic material as well as the location of abnormalities, many of which correspond to particular diseases or defects that are treatable with proper health care. Cytogenetic imbalances, gains and losses of the genetic material making individuals who they are, are the most frequently identified causes of mental retardation and developmental delay which affect 1-3% of children. These elements are often seen in conjunction with congenital anomalies, dysmorphic features, and growth retardation (Aradhya, Manning, Splendore, & Cherry, 2007).

Signature Genomic Laboratories has developed a new version of the microarray with increased resolution. The previous versions of the SignatureChip (versions 1.0 through 4.0) combined show a clinically relevant abnormality detection rate of 6.9% for all patients tested (Shaffer, Bejjani, Torchia, Kirkpatrick, Coppinger, Ballif, 2007). While a majority of these patients were tested with the SignatureChip Version 4.0, Version 4.0 was not evaluated individually. The increase in resolution should be evaluated to determine if the SignatureChip WG is effective in finding more genetic abnormalities. This gives Signature the ability to answer questions posed by the patients, their families, and the physicians involved, regarding the clinical management of the child's condition. This increase in knowledge is also beneficial to the company, providing the potential to take even more market share in this particular aspect of genetic testing.

The purpose of this study is to determine if increasing the resolution of the SignatureChip is associated with increases in the abnormality detection rate. The central hypothesis is that the increase in resolution is associated with an increase in the abnormality detection rate. The basis for this hypothesis is that genetic anomalies can exist in all regions of the human genome. If the resolution of the microarray is increased, therefore representing a larger portion of the human genome, more genetic abnormalities are detected. The technology has the ability to detect a gain or loss of genetic material in each region of the genome studied. When resolution is increased, and more specific regions of the genome are included on the microarray, the technology provides information about all of the new regions. An increased resolution allows the interpreter to see more abnormalities that were not detected with lower resolutions. This increased information will most likely correspond to an increase in abnormalities that were once undetected corresponding to an increase in the abnormality detection rate. While data are not published,

Signature Genomics has seen an increase in the abnormality detection rate with every increased resolution of the SignatureChip. One may infer that another increase in resolution is associated with yet another increase in the detection rate.

With the exception of discovering balanced rearrangements, the microarray technology is similar to conventional cytogenetics, also known as a karyotype. However, the primary advantage of the new technology is the significantly higher resolution (Aston, Whitby, Maxwell, Hair, Woley, Lowry, 2008). Routine cytogenetic analysis by GTG banding has the ability to detect DNA alterations, such as losses and gains, of greater than 3-5 million base pairs (Mb) (Shaffer & Bejjani, 2005). Alterations smaller than this are usually more difficult to visualize and often undetectable with conventional cytogenetics (Shaffer & Bejjani, 2004). Because the microarray enables visualization of the genome using comparatively smaller pieces of cloned DNA, resolution is dramatically increased from the standard cytogenetic karyotype (Bejjani, Saleki, Ballif, Rorem, Sundin, Theisen, 2005).

According to Bejjani et al. (2005), comparative genomic hybridization microarrays (aCGH) are developed for genome-wide screening of chromosome alterations by comparing differentially labeled test (patient) and reference (control) samples of genomic DNA. The microarray consists of thousands of large pieces of cloned DNA, such as bacterial artificial chromosomes (BACs), known to have a specific location within the human genome. These DNA segments are then spotted onto a glass slide to create an array. This development substantially increases the resolution, only limited by the size of the nucleic acid targets used as well as the distance between the clones (Pinkel, et al., 1998). Because of these capabilities, a comparison of ratios between overlapping clones may narrow the abnormality region to within a

fraction of the clone and determine the exact size of the copy-number variant (Bejjani & Shaffer, 2006). Because the microarray has the ability to scan the entire genome and not one specific area, as in fluorescence *in situ* hybridization (FISH), there is no need for a suspicion of a particular phenotype before testing (Kashork, Theisen, & Shaffer, 2008). These are substantial benefits of the microarray when compared to conventional cytogenetics. However, while appealing, an array covering the entire genome at a very high resolution has potential disadvantages for use in the clinical settings. These large, higher density arrays are expensive to fabricate, difficult to control for quality, and have a higher likelihood of showing significant levels of copy number variants (CNVs) making interpretation more difficult (Choe, Kang, Bae, Lee, Hwang, & Kim, 2007).

Chromosome analysis is a key approach to diagnostic studies of patients suffering from developmental delay, mental retardation, dysmorphic features, and birth defects (Bejjani et al., 2005). Imbalances in DNA, found using cytogenetic studies, often explain certain abnormal phenotypes and may correspond to specific syndromes or diseases (Bejjani et al., 2005). The most important goal of microarray testing within medical practices is to provide physicians as well as patients clinically useful results for diagnosis, genetic counseling, prognosis, and clinical management of cytogenetic abnormalities (Bejjani & Shaffer, 2006). The design of the microarray is very important for diagnostic setting use. The microarray provides distinct advantages over conventional cytogenetics in detecting clinically significant chromosomal abnormalities, specifically microscopic and submicroscopic abnormalities (Bejjani & Shaffer, 2006). In many cases, children present with features specific to a known syndrome or disorder providing the clinician with information to send for testing for a specific genetic disorder such as Down syndrome or DiGeorge syndrome. However, strong clinical suspicion of a specific

disorder is often challenging with neonates with birth defects because their clinical presentations are atypical or because their features are shared by several genetic disorders. Neonates may also lack specific features that do not develop until later in life (Lu, Phung, Shaw, Pham, Neil, & Patel, 2008). Microarray CGH provides clinicians with an all-inclusive test, enabling hundreds of tests for different genetic disorders to occur concurrently. This quickly identifies or eliminates most of the known chromosomal imbalance disorders in one test rather than hundreds of individual tests.

The identification of a chromosomal abnormality is critical to clinical management of the condition to not only anticipate potential medical problems, but also to understand recurrent risks for further offspring within the affected family. The identification of a pathogenic abnormality is likely to impact patient care since proper referrals to specialists or therapeutic interventions will minimize some of the known effects of these abnormalities. This may include screening for known anomalies like cardiac defects or early interventions for learning disabilities. For some families, the proper diagnosis diminishes anxiety and allows for risk recurrence estimates for future pregnancies (Edelmann & Hirschhorn, 2009). Most importantly, microarray testing is instrumental in providing a diagnosis for individuals suffering from syndromes not yet detected with other tests (Shaffer & Bejjani, 2006). However, many of these patients may have a normal result on the microarray for one of two reasons: (1) the patient has a microdeletion or microduplication that is not detectable due to the low resolution of the microarray, or (2) the patient does not have one of these abnormalities and suffers from something that this test cannot diagnose. Therefore, an increase in the microarray resolution has the potential to diagnose another subset of patients, those with a normal result on a previous version of the microarray.

Initially, the SignatureChip microarray contained a few hundred clones corresponding to locations on the chromosomes. This targeted microarray was specifically designed to detect unbalanced rearrangements of the subtelomeric regions of the chromosomes as well as other clinically significant regions. The chromosomal locations were carefully chosen based on their clinical significance and associated known phenotypes. Signature Genomics also paid close attention to the distance between the clones, hoping to reduce the gaps in coverage. With the combined SignatureChip Versions 1.0 – 4.0, the company detected genomic abnormalities in 6.9% of patients. These abnormalities include unbalanced rearrangements, microdeletions, microduplications, aneuploidies, and mosaic abnormalities.

Since the initial version of the microarray, the company has increased the resolution of the test by adding more targets and regions to test. The array now contains 4,600 bacterial artificial chromosome (BAC) clones and contigs of clones in every band at 850-band resolution. This corresponds to over 21.4% of the entire human genome. Over 150 recognized genetic syndromes are now covered. The new chip, SignatureChip WG, has thousands of clones and is a new service the company offers. Because of this increased resolution, Signature Genomics suspects that the abnormality detection rate will increase. One study shows that an increase in resolution, from 853 BAC clones to 1475 BAC clones with greater backbone coverage, increased the detection rate from 13.7% to 16.6% (Lu et al., 2008). However, the patients tested using the two versions of the BAC microarray were different groups of patients, so the increase in detection rate may not be entirely reflective of an increase in genome coverage. Increasing resolution of the microarray is a continual process, and Signature Genomics has found detection rates from the previous version and is developing these rates for the new version. If the increased resolution is associated with an increase in the abnormality detection rate, then the

company, as well as others in the field, may consider further increases in resolution to obtain an even higher rate.

Research question

The research question is: Is the increased resolution of the SignatureChip WG associated with an increase in the abnormality detection rate of the laboratory's patients? Answering this question will allow Signature Genomics to determine whether future endeavors in increasing resolution on the SignatureChip microarray may benefit the patients seeking testing.

CHAPTER TWO

LITERATURE REVIEW

Although microarray technology is a rather new development, it has grown in popularity quickly with many laboratories around the world discovering the advantages of such technology. However, many laboratories are still using microarrays for research purposes and have not yet advanced to clinical use. As studies are published, the majority of the results show a great amount of support for the clinical use of comparative genomic hybridization as a diagnostic tool. CGH microarray analysis is rapidly becoming a mainstream clinical diagnostic test, and further studies will aid in the clinical acceptance of the technology.

Methodology used in conducting the literature review

The primary database used for this literature search and review is PubMed. Using this database and key words "resolution" and "clinical microarray" as well as "detection rates" and "copy number variants," a variety of peer-reviewed articles are evaluated. No time limit is set for the literature search since this is a relatively new technology. Relevant articles had to discuss microarrays in the clinical setting for purposes of detecting genetic abnormalities. Those discussing cancer cases as well as other uses of the microarray are excluded from the review. Another inclusion criterion is the use of BAC arrays rather than oligonucleotide or other types of arrays since the SignatureChip microarray is a BAC array.

With the key words "resolution" and "clinical microarray," PubMed identified one hundred nine articles. Of these, many discuss other uses of the microarray not pertaining to genetic testing and are eliminated from the review. This search provided seventeen relevant

articles. The next search of "clinical microarray" and "detection rates" resulted in eight articles, two of which are included in this review. The final search performed was "clinical microarray" and "copy number variants" resulting in a list of six articles narrowed to three with the inclusion and exclusion criteria. Overall, twenty-two articles are included in the following literature review.

Key literature review findings

Microarray vs. conventional cytogenetics

Many cytogenetists and physicians have observed the power of microarray testing compared to routine cytogenetics testing (a karyotype) and/or FISH. Traditional study of human chromosomes with banding techniques, karyotyping, has been the test of choice for over thirty years in defining large-scale genomic changes (Shaffer & Bejjani, 2006). The 1990s brought a new type of technology, florescence *in situ* hybridization (FISH), advancing the ability to detect known syndromes and identify chromosomal rearrangements. This technology allows clinicians to request testing when patients present specific clinical phenotypes suggestive of a particular disorder. The probes used for this approach cover the commonly deleted regions observed for particular syndromes but may miss smaller or atypical deletions. The need to screen the entire genome for gains and losses of material, however, has led to the discovery of microarray-based comparative genomic hybridization (Edelmann & Hirschhorn, 2009).

Other applications of FISH include aneuploidy screening in prenatal specimens and rearrangements of the subtelomeric regions (Shaffer & Bejjani, 2006) Subtelomere FISH is recommended for the investigation of children with unexplained mental retardation or developmental delay, and studies have shown the detection rate of ~2.5% in diagnosing

subtelomere abnormalities. However, in most cases, this testing does not identify the size of the abnormality or complex subtelomeric rearrangements. Expansion of the subtelomeric regions with microarray technology allows for more complete characterization of imbalances as shown in 56% of cases with subtelomeric abnormalities tested on an increased subtelomeric resolution microarray (Ballif, Sulpizio, Lloyd, Minier, Theisen, Bejjani, & Shaffer, 2007). Subtelomeric abnormalities are some of the more common chromosomal aberrations. Shao et al. (2008) found a high frequency of subtelomeric rearrangements, 499 out of 5,380 patients (9.3%) demonstrated these types of abnormalities. Microarray technology is the most efficient way of displaying these types of chromosomal imbalances.

Two hundred sixty-four clinical cases are examined by Choe et al. (2007) to compare microarray results with conventional cytogenetics results. All but one case, a 47,XXX, have array results in exact accordance with the cytogenetic results. The one case of sex chromosomal polysomy was not detected using array CGH. However, changing the reference sample used to DNA from a 47, XXY reference sample will eliminate this limitation (Choe et al., 2007). A study by Aston et al. (2008) shows an abnormality rate of 10.8% of patients with normal karyotype reports. These abnormalities are all confirmed with a FISH test. Within this same study, all cases that are reported to have an abnormal karyotype are confirmed with the microarray as the same abnormality after excluding those cases with balanced rearrangements. Bejjani et al. (2005) also find that known abnormalities from previous karyotype reports are detected with array CGH and confirm this with 94% (34/36) of known abnormalities. The two cases that did not fully correlate with the karyotype actually discovered errors in the original report displaying the increased sensitivity of the newer diagnostic test. Bar-Shira et al. (2006)

finds novel chromosomal aberrations in two of five, 40%, previously undiagnosed patients using CGH microarrays.

In a study done by Thorland et al. (2007), ten normal patients along with sixty-seven patients with subtelomere anomalies previously confirmed by cytogenetic studies are analyzed using array CGH. All ten normal patients and 97% (72/74) of the abnormal patients are correctly interpreted. One patient's analysis detects a second abnormality not previously found. The two patients with 20q deletion or duplication, a loss or gain of genetic material on the long arm of chromosome 20, previously detected did not demonstrate the abnormality on the array possibly because of lack of coverage in that region. Pickering et al. (2008) finds an increased detection rate of 7.9% in cases that were tested using array CGH that had either no cytogenetics performed or had a normal cytogenetics report.

Overall, the microarray diagnostic test has not only proven as effective as conventional cytogenetics but also has detected further abnormalities. There is another advantage of time saved using a microarray as a diagnostic tool instead of the karyotype. Conventional cytogenetics is a very labor intensive process that may take as long as twenty-one days to deliver a result. This is due to the culturing of the cells as well as the tedious work it takes to examine every chromosome for subtle structural differences. On the other hand, array CGH takes as short as 48 hours to report findings from the test. This is a great advantage especially when considering babies in the neonatal intensive care unit or children waiting for surgery.

These studies display the power of the microarray technology combined to the array CGH. Array CGH is able to detect smaller chromosomal abnormalities than the typical karyotype which is historically the chosen test for patients presenting indications of genetic

abnormalities. This leads to the suggestion that many conventional cytogenetic laboratories in the clinical field will introduce this diagnostic tool into their own repertoire of tests offered to patients. Within the next few years, it is likely that the microarray will replace the traditional karyotype as the initial investigation of chromosomes (Bejjani et al., 2005).

New discoveries

While confirming and clarifying karyotype results is significant, finding undiscovered chromosomal abnormalities is the more likely result when an abnormality is detected. In one of the largest published studies using a targeted microarray, 1,500 clinical samples are analyzed for chromosomal abnormalities due to developmental problems. These cases are consecutive and in an unselected population. In this group, 5.6% of patients show a clinically relevant abnormality, previously unknown, and many had normal karyotype reports along with other more specific diagnostic testing. Of these abnormalities, known microdeletion syndromes, such as DiGeorge syndrome, are among the most common abnormalities identified indicating that the clinical phenotype for these patients is more varied than previously thought (Shaffer, Kashork, Saleki, Rorem, Sundin, Ballif, 2006).

Another more exciting prospect of the microarray technology is the discovery of new syndromes not previously described. Because the microarray has a much higher resolution than previous tests, it has the ability to find abnormalities once thought to not exist due to the small size of the alteration. A cytogenetic basis for a microdeletion syndrome is the identification of a common deletion in a cohort of individuals with similar phenotypic features. Once the syndrome is well described, physicians can return to patients with similar phenotypes and re-examine their chromosomes looking for the described deletion (Shaffer, Theisen, Bejjani, Ballif, Aylsworth,

Lim, 2007). This is the method of microdeletion syndrome discovery. However, a single case report can lead to the discovery of a new syndrome if documented and thoroughly studied. The introduction of the microarray has aided in discovery of such novel syndromes. Combining the new technology with the old way of identifying microdeletion syndromes has the ability to uncover the chromosomal basis of syndromes quickly (Shaffer et al., 2007).

Recently, with the use of array CGH in large numbers of patients, new syndromes were discovered and characterized. These include the deletion of 1q41q42 (Shaffer et al., 2007, Genetic Medicine), deletion of 16p12 (Ballif, Theisen, McDonald-McGinn, Zackai, Hersh, Bejjani, 2008), and deletion of 17q21.3 (Sharp, Hansen, Selzer, Cheng, Regan, Hurst, 2006). After comparing two unrelated cases with *de novo* 9q22.32-q22.33 microdeletions, several common features emerge including trigonocephaly, overgrowth, and small mouth signifying a new syndrome within a region already characterized. While these two cases have a chromosomal aberration within a known region, the size of their abnormality dictates different characteristics from those known to have larger deletions which may correspond to a different management of care (Redon, Baujat, Sanlaville, Le Merrer, Vekemans, & Munnich, 2006). As with other new syndrome discoveries, patients suffering from these specific chromosomal alterations now have definitive clinical knowledge about the reason for their indications. Physicians of these children are able to communicate with each other to better characterize these new syndromes providing much needed information to the patients and families, including future medical endeavors resulting from the syndrome.

Copy-number variations

The previous studies show significant evidence that the microarray test in clinical settings is extremely useful in diagnosis of chromosomal abnormalities. However, when considering the increase in resolution, one of the main concerns is the increase in unclear copy number variants (CNVs). A CNV is operationally defined as a "DNA segment, longer than 1 kilobase, with a variable copy number compared with a reference genome" (Lee, Iafrate, & Brothman, 2007, p.48). In simpler terms, a CNV is a segment of DNA, at least one kilobase long, within a chromosome that has either a gain of material (a duplication) or a loss of material (a deletion) when compared to a known normal reference sample. While many CNVs correspond to well-understood syndromes, there are many that are not clinically relevant and abundantly seen in the normal population, (de Stahl, Sandgren, Piotrowski, Nord, Andersson, Menzel, 2008; Korbel, Urban, Grubert, Du, Royce, Starr, 2007; Lee, Iafrate, & Brothman, 2007; Perry, Ben-Dor, Tsalenko, Sampas, Rodriguez-Revenga, Tran, 2008; Pinto, Marshall, Feuk, & Scherer, 2007). In fact as of 2007, 18.8% of the euchromatic human genome is annotated as copy-number variants (Pinto, Marshall, Feuk, & Scherer, 2007). These CNVs may play a significant role in the phenotypic variation of the human population (Korbel et al., 2007). That is, they may explain why each individual is different in looks and other characteristics from others.

This abundance, however, often makes it difficult to determine if such variations are clinically relevant, the explanation of why the patient has abnormal development or features. There are concerns about the difficulties in accurately and efficiently discriminating between pathogenic CNVs and benign CNVs in the human genome. One study reports CNV frequencies ranging from 2.6% to 35.1% of the population tested (Thorland, Gonzales, Gliem, Wiktor, &

Ketterling, 2007). After having identified CNVs by microarray analysis, the first step is to discriminate between common CNVs and rare CNVs since it is unlikely, though not completely understood, that CNVs frequently found in a normal population are directly related to clinical diagnoses like mental retardation (Koolen, Pfundt, de Leeuw, Hehir-Kwa, Nillesen, Neefs, & Scheltinga, 2008). When CNVs are discovered, clinicians must pay attention to whether such a deletion CNV is found in healthy control individuals as well as the size of the deletion. By studying large normal cohorts and unaffected parents, disease-causing CNVs are distinguished from variations without clinical significance (Koolen et al., 2008). Larger deletions may be clinically relevant even though similar CNVs exist in the normal population (Lee, Iafrate, & Brothman, 2007). In a study performed by de Stahl et al. (2008), a total of 87% of the CNV regions reported overlapped with genes. This overlapping may disrupt genes or alter gene dosage which in turn will affect gene expression and can have phenotypic consequences. Increasingly smaller CNVs are detected with the onset of high-resolution microarray platforms. Interpretation of all CNVs is a complex process and may hamper the clinical interpretation due to the complexity of the human genome (Koolen et al., 2008). Further research into the discovery and characterization of CNVs is needed to develop more comprehensive genetic maps. This will allow for more accurate reporting and interpretation as well as increased knowledge of human CNVs (Lee, Iafrate, & Brothman, 2007).

The introduction of microarrays into the clinical setting has proven as effective, if not more effective, in detecting gains or losses in patients' genetic makeup. Not only are known abnormalities found, but novel abnormalities are also discovered. While this technology continues to show promising advances in genetic diagnostic testing, analysts must pay particular attention to unclear copy number variants. These are common in the normal population and too

much reliance on them results in inaccurate reporting. It is evident that this technology continues to rapidly advance the field of cytogenetics.

Gaps identified in the literature

While there is literature discussing the effectiveness of the microarray technology as opposed to conventional cytogenetics, there is no study specifically focusing on the difference between two versions of a microarray, one with higher resolution. Shaffer et al. (2007) describes the combined abnormality detection rates of the previous versions of the microarray. This study, however, compares only two versions of the SignatureChip and explicitly examines the association between the increased resolution of the SignatureChip WG and the abnormality detection rate.

There are many other gaps in the literature relating to the use of microarrays in the clinical setting. It is essential to further study CNVs and their impact on clinical diagnosis especially in relation to mental retardation (Koolen, 2008).

Potential contribution

Targeted arrays will continue to require updates in order to stay current with newly reported novel syndromes associated with regions of the genome (Bejjani & Shaffer, 2006). This combined research will provide knowledge about newly discovered copy-number variations in the normal population excluding those from clinically relevant genetic alterations. The eventual eliminations of clinically normal variants from microarray tests may result in clearer interpretations. As the cytogenetic field continues to learn more about the genetic code, it is able to discover new syndromes or diseases and learn how to better treat patients suffering from these

defects. An increased resolution allows diagnostic analysts to see the genetic material that was once impossible to see, ultimately leading to the possibility of discovering what is "wrong" with the patients that are tested. The availability and compilation of this information enable the grouping of patients according to the findings. The display of similar characteristics by these patients may help define new syndromes or diseases. These discoveries give patients answers addressing what they are dealing with as well as information to parents concerning whether they may conceive another child with the same disorder. If increasing the resolution of the SignatureChip is associated with a discovery of a new disease, more patients and families will have a clearer clinical treatment plan potentially increasing their functionality. Signature Genomic Laboratories and other companies within the field will continue to develop new technologies that increase the resolution and the ability to detect abnormalities. There will, however, come a point at which the increased information is no longer meaningful due to further discoveries of normal copy number variants and not worth the endeavors, time and money, needed to develop it. This study takes current knowledge one step further in trying to discover the optimal resolution for genetic testing.

CHAPTER THREE

METHODS

This section describes the methods used to answer the research question. It details the population, sample, and the criteria for inclusion in the study. The study is outlined and the hypothesis is discussed including a detailed description of the source of data. A discussion of the variables and the analytic method are also included.

Population and sample

Signature Genomic Laboratories has tested over twenty-five thousand patients in the four years of clinical diagnostic testing and continues to see an increase in number of patients needing diagnostic testing. These patients live in all areas of the world and suffer from an array of symptoms that lead physicians to suspect some type of chromosomal abnormality. The majority of patients suffer from some form of developmental delay, dysmorphic features, seizure disorders, or mental retardation. While most of the patients are infants or children, Signature Genomics tests patients of all ages, anywhere from one day old to seventy years old. The population of interest for this study is the group of patients tested using both the SignatureChip Version 4.0 microarray and the SignatureChip WG microarray. All patients tested during the period of May 2006 through October 2007 were evaluated with the SignatureChip Version 4.0, and those patients tested November 2007 through June 2008 were evaluated with the SignatureChip WG. However, physicians have the ability to request a diagnostic test upgrade using the new version of the microarray for a patient previously tested on the older version. For this study, the population of interest is the group of patients tested on both platforms. In order to

determine whether the sample sizes for these subgroups are appropriate for the statistical tests chosen, a power calculation is performed using G*Power 3.0.10 (Faul, Erdfelder, Lang, Buchner, 2007) prior to any statistical analyses.

Study design

This is a comprehensive, retrospective study of the medical records of the patients tested using both versions of the microarray. For the purposes of this study, secondary data collection, through the use of Laboratory Information Management System (LIMS) electronic reports, takes place within Signature Genomics headquarters. While secondary data collection is not ideal in some research settings, it works for this study due to the availability of the needed information in the patient electronic reports.

A review of every electronic chart of the entire population takes place analyzing both the Version 4.0 and WG microarray data as well as any other pertinent information such as whether FISH confirms the abnormality, whether parents were tested, and the patient's age when tested. Within the laboratory, a visualized abnormality is confirmed using a different technology, FISH, before it is accepted as a true abnormality and reported to the ordering physician. This information is also available in the patient's records and is a great source of abnormality history. Ultimately, the laboratory director has the final decision whether a copy number variant is reported as a chromosomal abnormality and then provides the written report. If a patient is found to have an abnormality and it is of unknown clinical significance (that is it is not in a region of a known genetic disorder) then the patient's parents are often tested to determine whether the abnormality was inherited from one of them or considered *de novo*, a genetic

abnormality that neither parent possessed or transmitted. Related cases are noted in each of the patient's charts.

Using this available information, the researcher is able to analyze the electronic report from the Version 4.0 and WG microarrays. All pertinent information for each patient is accumulated into a large data set. This includes the patient's age when the test was requested, the indication for study, the test results for both of the microarrays, the confirmation of the abnormality, and whether the parents were tested which will aid in determining whether the abnormality is of clinical significance or an insignificant copy number variant. The patient's accessioning number suffices as an identifier and no names are used. With all information in one location, the researcher has the ability to determine the detection rate of the new version, SignatureChip WG, and compare it to the older version, SignatureChip 4.0.

Data sources and method of data collection

As a patient's sample arrives at Signature Genomic Laboratories, an accessioning number (SGL number) is assigned. This provides double identifiers for every patient and either the name or the SGL number is used when discussing results or other personal information. All employees of Signature Genomic Laboratories are able to access detailed information pertaining to the patients that are tested. Data from Signature Genomics' patients are available both paper-based in patient files as well as electronically through the internal Laboratory Information Management System (LIMS) and Genoglyphix database. All information is protected with either locks or electronic passwords. The LIMS system contains all information relevant to the patient and follows the patient through the various stages of the laboratory. Information such as name, date of birth, physician, institution, and a copy of the report the physician received is available

through this dense database. Genoglyphix stores the raw data that each individual test obtains as well as the graphs and plots used to determine whether an abnormality exists on any of the patient's chromosomes.

As the test is conducted, the patient's color-coded chart is passed from one process to the next following the laboratory's procedure. Data are added at every step, and hard copies of the graphs generated are included. The final report that is sent to the physician is also added to the chart before it is filed. If more than one test is performed, information is entered into both the database and the paper-based chart for each of the tests. This study will use the laboratory's information system and the electronic reports to evaluate the differences between the SignatureChip Version 4.0 microarray and the SignatureChip WG microarray.

Because Signature Genomic Laboratories is a fee-for-service laboratory that obtains consent to perform the microarray test, this study uses the available result data as secondary data, and all information is coded according to the patient's accessioning number (SGL number), no further consent is needed to perform this type of study. All samples are de-identified and no patient's identifiers will be used in any publication. The researcher secured an expedited review through the WSU IRB on the basis of de-identification of archived specimens. Confidentiality is followed throughout the study by using only the SGL numbers as patient identifiers. All information is kept in locked file cabinets as well as electronic databases protected by passwords. Due to SGL's role in healthcare, the company takes confidentiality very seriously and does not share information with physicians other than the patient's own physician.

Study variables

The dependent variable in this study is the abnormality detection rate for each of the versions of the microarray. The independent variable of interest is the version of the microarray used, either Version 4.0 or WG.

The population has results for two tests, both the 4.0 and WG versions of the SignatureChip. All samples also have confirmatory test results with an independent method (FISH). Both microarrays are distinct tests, with the WG having the same backbone as the Version 4.0 microarray but also an increase in clone numbers resulting in a greater resolution. These tests are developed within Signature Genomics and do not vary in any aspect. There is a set template for both of the microarrays and no variation occurs patient to patient. All patients are tested using the same template no matter the age or indication for study of the patient. This, in itself, is a great source of reliability and internal consistency. The testing conditions are standardized for the entire population of Signature Genomics' patients.

Those copy number variants, different amounts of DNA than the control, that do not reach the significance level (0.3) are ignored and treated as normal variation. When an abnormality is significant, further testing known as FISH is performed on that patient's sample. If the abnormality is a microdeletion, a confirmatory FISH result includes five metaphase cells, cells that are in a dividing phase of the cellular cycle, distinctly showing the abnormality. This is displayed through a microscope with one of the chromosomes exhibiting two signals, the control which is colored green and the target which is colored red, and the other chromosome only exhibiting one signal, the control (green) with no target signal. The confirmatory FISH process is somewhat different for microduplications. Again five metaphase cells are analyzed looking

for a possible three target signals (red) rather than the normal two signals. Fifty interphase cells, those cells in the resting phase of the cell cycle, are also analyzed noting those cells that contain three red signals and those that contain two red signals. In this case, 70% of the interphase cells must show three signals in order for the FISH test to confirm the original microarray test. For chromosomal rearrangements or marker chromosomes, the same process as microdeletions is followed with five metaphase cells analyzed. This process of FISH confirmation is an important step in reporting the chromosomal abnormalities to the patients and their physicians.

Analytic methods

The gathering of this data results in a specific abnormality detection rate for the SignatureChip Version 4.0 and a detection rate for the SignatureChip WG, or a proportion of abnormal patients in each of the testing categories. The two proportions are compared using McNemar's test for correlated proportions with a degree of freedom of 1 and an alpha of 0.05. While chi square tests the hypothesis that proportions estimated from two independent samples are equal, McNemar's test allows for the situation of matching samples that are not independent and for testing whether there is a significant change between before-and-after situations. With this, McNemar's test assumes no change in the proportion of patients found to have genetic anomalies between the two versions of the microarray.

$$\text{McNemar's chi square test: } \chi^2 = (b-c)^2/(b+c)$$

where b and c represent the two categories where change occurred (Kuzma & Bohnenblust, 2001).

Observed frequencies are entered into a two by two table with each quadrant representing a normal and abnormal combination. The top of the chart corresponds to the reference category, in this case Version 4.0 microarray, while the side of the table corresponds to the index category, in this case WG microarray. Each of the microarrays has two possible outcomes, normal or abnormal results. The figure below displays this.

		Version 4.0 Result	
		Normal	Abnormal
WG Result	Normal	a	b
	Abnormal	c	d

Figure 1: McNemar's chi square test two by two table. Quadrant a represents the number of cases that had normal results for both versions of the microarray, and quadrant d represents the number of cases that had abnormal results for both versions of the microarray. The two quadrants of interest are b and c where there is a difference in results from one test to the next.

The McNemar's test determines whether the difference in proportions is statistically significant or not (Kuzma & Bohnenblust, 2001).

Once the data are collected and entered into a table similar to the one above, the statistical software package SAS is utilized to perform the statistical analysis. The following program provides SAS with commands to run McNemar's test for correlated proportions and produce an output that evaluates the differences between the two tests.

```
data testresult;
   input case control y;
   datalines;
   1 1  d
   0 1  b
   1 0  c
   0 0  a
   run;
 proc freq data=testresult;
  table case*control ;
  exact mcnem;
  weight y;
 run;
```

where a, b, c, and d represent quadrants as stated above, with zero representing a normal result and one representing an abnormal result. When entering the data obtained into the two by two table discussed above, a = 156 patients, b = 0 patients, c = 32 patients, and d = 24 patients.

The statistical output denotes the chi square value as well as a corresponding p value which indicates the likelihood whether the difference between the two microarray proportions is statistically significant.

CHAPTER FOUR

RESULTS

A query of SGL's internal Laboratory Information Management System (LIMS) found 218 patients that were originally tested for genetic aberrations using the SignatureChip Version 4.0 microarray but also had follow-up testing using the newer, higher resolution microarray, SignatureChip WG. Those patients requesting microarray testing but already known to have a genetic abnormality from other testing were immediately excluded from the data set since the microarray was not the primary test for diagnosis. Of the 218 patients, six were known to have genetic aberrations leaving 212 patients for the analysis. Using G*Power 3.0.10 (Faul, Erdfelder, Lang, Buchner, 2007) with an alpha of 0.05, an effect size of 0.4769534 (obtained from G*Power using the Version 4.0 normal and abnormal rates as H0 and WG normal and abnormal rates as H1), and a power of 0.95, the total sample size needed is 58 patients. The total population of 212 is considerably higher than this value and should result in an accurate McNemar's chi square test.

All patients were first tested using the SignatureChip Version 4.0. Twenty-four of the 212 patients had abnormal results using this microarray corresponding to an 11.3% abnormality detection rate. These abnormalities ranged in size as well as location within the genome. As requested by the patients' physicians, these patient samples had follow-up testing with the newer version of the microarray, SignatureChip WG. Of the 212 patients, 56 were found to have abnormal results, resulting in an abnormality detection rate of 26.4%.

Every patient with abnormal results using SignatureChip Version 4.0, a total of 24, also had abnormal results with the SignatureChip WG. The higher resolution microarray confirmed the results from the early version in all cases but also better defined the abnormalities in some cases or found new abnormalities in other cases. Of the 24 originally abnormal cases, 11 were confirmed the exact same size of the Version 4.0 aberration with the SignatureChip WG. The SignatureChip WG confirmed and better delineated the abnormality in 5 of the cases. Three cases showed the same original abnormality of the same size but also showed a new abnormality with the increased resolution. The SignatureChip WG results for 2 of the original 24 abnormal patients report a better delineated Version 4.0 abnormality as well as a new abnormality discovered by SignatureChip WG. Two of the original cases showed two abnormalities on the SignatureChip Version 4.0, and the SignatureChip WG better delineated both abnormalities. The last case of the 24 cases had three abnormalities on the original microarray, and all were confirmed and better delineated using the SignatureChip WG.

With the increase in resolution, the SignatureChip WG detected abnormalities in an additional 32 patients, a total of 56 patients. The results of SignatureChip Version 4.0 are 24 abnormal patients and 188 normal patients. The additional resolution microarray provided 56 total abnormal patients and 156 normal patients. Using the SAS software and the written program, these results lead to a McNemar's chi square value of 32.0 with an associated p value of less than 0.001 and one degree of freedom. The difference between the abnormality detection rates of the two microarrays, as denoted by the p value, is considered to be statistically significant. The increase in resolution of the SignatureChip WG is associated with an increased abnormality detection rate.

CHAPTER FIVE

CONCLUSIONS

This large study of 212 patients, tested using both the SignatureChip Version 4.0 and SignatureChip WG platforms, has assessed the clinical utility of increasing the resolution of targeted array CGH testing. The results of the McNemar's chi square test confirm that an increase in resolution of the microarray is associated with an increase the abnormality detection rate. Not only is there a significant increase in detecting abnormalities with the higher resolution microarray, but the new version was also able to better characterize the abnormalities originally found using the Version 4.0 platform in a majority of the cases.

Biases

Due to the extensive cost of this test, not all patients originally tested on an earlier version of the microarray are retested when a new, higher resolution, microarray is released for clinical use. However, physicians are able to request an additional test using the high resolution microarray if they would like to pursue further testing. Most patients that fall into this category are patients with a normal result on the initial array. Therefore, the set of patients with results on both Version 4.0 and WG may have some existent bias resulting in a potentially lower abnormality detection rate for Version 4.0 and a more significant difference in abnormality detection rates between the two platforms. However, the p value of less than 0.001 associated with the chi square shows that there is clearly a very significant difference between the two abnormality detection rates, and with the elimination of the bias, results would most likely

continue to show a significant difference since the abnormality detection rate for Version 4.0 would be lower while the detection rate for WG would be similar to the rate found in this study.

Limitations of the study

While the findings of this study are fairly conclusive, there are limitations to the study. Only patients tested using both platforms are analyzed limiting the number of patients to those with specific requests for further study. A larger, more comprehensive study will provide more accurate abnormality detection rates for each of the two platforms and a more accurate difference between the two detection rates. Array CGH does not have the ability to detect balanced translocations and inversions because of the lack of genetic imbalance. These types of aberrations may exist in the patients' genetic material but are not detected and diagnosed as abnormalities. Copy number variants (CNVs) of unknown significance are often included as abnormalities in the issued report. Existence of these will inflate the abnormality detection rate. However, both versions of the microarray are likely to have the same inflation rate which does not affect the difference between the two platforms. The most evident limitation of this study is the resolution of the microarray. The SignatureChip WG has a much higher resolution than the SignatureChip Version 4.0, but there is no information about the abnormality detection rate with additional increases.

Due to the process of the technology and the characteristics of human DNA, the microarray does not produce false positives or false negatives other than the potential diagnosis of an aberration due to an unknown or not well studied CNV. So, directors responsible for reporting results must fully rely on the knowledge of CNVs and their locations within the genome.

Implications for patients and their clinicians

The microarray technology, in and of itself, has great implications to the patients and families suffering with mental retardation, developmental delay, or multiple congenital anomalies. Array CGH is much quicker and more objective than the conventional forms of cytogenetics such as karyotyping and FISH. Hundreds of syndromes are tested concurrently rather than examining one at a time. With higher resolution arrays, new disorders are discovered providing patients with information about their indications and how to properly seek medical or therapeutic care to limit the effect of the genetic disorder on their lives.

Physicians and other clinicians must also educate themselves on the availability of array CGH and the release of higher resolution, more advanced versions of the tests already performed with the patients' genetic material. A normal result on a report does not eliminate the possibility of a chromosomal imbalance. A smaller microdeletion or microduplication may exist that is not detected with the platform of choice, but a platform with higher resolution may have the ability to detect the subtle aberration. A small deletion within a region of a clinically recognized disorder has the potential to present with much different features than once thought to exist as in the case of the 9q22.3 microdeletion overgrowth syndrome (Redon et al., 2006).

Genetic counselors work closely with physicians, patients, and their families. These individuals must also keep up to date with the technology, new disorder discoveries, and newly defined CNVs. Families rely on information from genetic counselors in discussing medical and therapeutic treatments and risk of recurrence with future pregnancies. Counselors must know everything that the microarray entails and share this with their patients in order to help them make the best decisions possible regarding the future.

Implications for Signature Genomic Laboratories

It is critical for Signature Genomic Laboratories to continue to refine and advance their understanding of this technology and others to keep up with the rapidly evolving field of cytogenetics. An increase in resolution will almost always result in an increase in CNVs. Signature Genomics has dedicated many resources to evaluating the significance of many of the CNVs by developing a database to track the recurrence of CNVs as well as testing parents of children found to have a copy number variant of unclear clinical significance. With the continued increase in resolution of the microarray, this process must persist and build upon information already known.

With the information from this study, another key implication for Signature Genomics is the continual education of its clients, all types of clinicians. The increase in resolution has a great impact on the amount of information a clinician is receiving with the final report. There are more areas of the genome covered and a higher likelihood that a CNV is found. This information is not always acknowledged by clinicians unfamiliar with the technology and may misinterpret the meaning of a normal result or a result of unclear clinical significance.

Signature Genomics' strategic plan may now include another further increase in resolution since it is evident that the increase from Version 4.0 to WG is beneficial in diagnosing a greater number of patients with chromosomal abnormalities. Another increase in resolution has the potential to affect the abnormality detection rate yet again.

Implications for health policy and administration

The available literature shows that array comparative genomic hybridization (aCGH) is a more effective test to find genetic aberrations than tests developed in the past. The microarray has the potential to find abnormalities at a level of detail once only dreamed of. This is true of many tests in the medical field. New technologies can prove as effective or more effective when studied accurately. However, the same tests may prove ineffective. The cost of this test may deter physicians and insurance groups from testing patients with clinical relevance. The amount of information obtained from microarray testing compared to conventional cytogenetics is considerable as is the cost difference. Health policy and administration must continuously evaluate the efficiency of technology and tests available for clinical use.

As genetic information becomes more available due to tests such as the microarray, health policy must determine whether more legislation must exist to limit the use of such information for discrimination. Privacy and confidentiality are often applied to genetic information in a stricter sense than other health information. The government must decide whether or not genetic information should be treated with greater priority and then pass legislation protecting the information in the way it should be protected. Copy number variants in the normal population may have an impact on a large group of people if not properly defined and accounted for under law. If this information is readily available to employers or insurers, some degree of discrimination may occur. However, the United States has already passed a law, Genetic Information Nondiscrimination Act of 2008, to protect employees and those being insured from this type of discrimination. More legislation may need to be passed if this law is not protection enough.

There are a variety of other legal and ethical issues related to genetic testing and the use of microarray technology. The organizations involved in genetic testing, including Signature Genomic Laboratories, may be held liable for legal issues regarding the increase in resolution of the microarray which would provide patients with more information regarding their genetic make-up. Ethical issues include whether to test patients using a new version of the microarray due to the increased likelihood of finding a novel abnormality not previously found. Commercialization of genetic research is another area of legal issue that genetic testing organizations must be in accordance with including intellectual property and existing patents. Legislation may determine that this commercialization should no longer apply to genetic testing and the products related to genetic material. Overall, there are many questions to continue to investigate and possibly legislate relating to genetic testing.

Final thoughts

The evolution of this technology is inevitable. As Shaffer and Bejjani (2006) state "The expectations of clinical cytogenetics from the medical community have been raised even higher as array CGH becomes part of the cytogeneticist's tools to examine chromosomes at an unprecedented resolution" (p 308). Clinicians will continue to ask for further increases in resolution until no further relevant information is gained with an increase in the resolution of a microarray. Given the ability to comprehensively detect chromosomal imbalances at a resolution unattainable by any other clinical test, array CGH with its continual increase in resolution will become the routine test of choice in diagnosing patients with symptoms of genetic disorders (Bejjani et al., 2005).

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