Blinded Fluorescence *In Situ* Hybridization Interpretation by Traditional Microscopy and Digital Image Capture Methods

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Abstract

The study aimed to compare two signal interpretation methods for identification of chromosomal abnormalities in patients. The methods used were traditional microscopy (TM) and digital image capture (DI) regarding fluorescence *in situ* hybridization (FISH) for detection of chromosomal abnormalities in cancerous cells. This research was a retrospective analytic, blinded study. The data were selected from cancer cases that were submitted to the Institute of Pathology (IOP), Ministry of Public Health, Thailand, in 2016. Totally, there were 40 cases used in the study, which can be separated into 30 cases of HER-2 (breast cancer), 5 cases of EWSR1 (sarcoma) and 5 cases of ALK (lung cancer). The data were analyzed using paired *t*-test to compare the performance of TM and DI methods. The results showed a high degree of concordance between them. This implied that both methods had similar efficiency in detecting HER-2, EWSR1, and ALK of FISH signal interpretation analysis. However, using DI method seemed to be more convenient and effective regarding human usage.

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Introduction

The molecular cytogenetic method of fluorescence *in situ* hybridization (FISH) has been globally adopted since the 1980s for its highly specific binding to the chromosome sections exhibiting great levels of complementary sequences.[1] This technique is useful for identifying specific DNA features and is used in a range of medical and genetic fields, including the diagnosis of cancer, by outlining the spatial-temporal gene expression patterns existing within tissues and cells.[2]

Following numerous international laboratories and institutes, Institute of Pathology (IOP), Ministry of Public Health, Thailand, has also begun to incorporate the FISH technique in investigations, particularly those concerning the diagnosis of chromosomal abnormalities in cancer via the traditional microscopy (TM) signal interpretation method. It has been heralded as a breakthrough discovery in translational imaging. With the advent of more specific and sensitive molecularly targeted diagnostic and therapeutic agents on the horizon, this technique is adopted worldwide and has significant potentials for advancement in the diagnosis.

Nevertheless, the IOP has recognized that the TM signal interpretation method appears to have certain limitations as well as specific disadvantages, which together implies that it is not an entirely accurate and reliable method for cancer detection. The main limitation of TM is fading of FISH signals, which means that a pathologist or technician would need to interpret and report immediately for an accurate result. In this regard, the present day digital photography may have the advantage because the images produced do not fade over time. This prompted necessity of this research: The goal was to investigate a potential alternative imaging technique which could be more efficient than the currently adopted TM signal interpretation method. Obviously, any alternate imaging method must be able to compensate for the drawbacks encountered while performing TM technique alongside FISH.

Our objective was therefore to statistically compare between TM and DI methods in regard to using FISH for detection of chromosomal abnormalities of cancerous cells.

Materials and Methods

The present study is a retrospective analytic research, which is a standard longitudinal study form often used in medical research. As such, the study compared two separate groups of diagnostic imaging modalities, TM vs DI methods. Both are similarly used alongside FISH to confirm chromosomal abnormalities of a cancer diagnosis, but differ by a certain characteristics, i.e. different techniques and imaging modalities.

Samples

Cases in this study were drawn from currently available data files, recorded and submitted to the IOP during the year 2016. Each section was

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Table 1 Criteria for interpreting HER-2[6]

Results	Single-probe average HER-2 copy number	Dual-probe HER-2/CEP17 ratio		
Positive	≥6.0 signals/cell.	≥2.0 with an average HER-2 copy number ≥4.0 signals/cell	≥2.0; with an average HER-2 copy number <4.0 signals/cell	<2.0; with an average HER-2 copy number ≥6.0 signals/cell
Equivocal	≥4.0 and <6.0 signals/cell	< 2.0 with an average HER-2 copy number ≥4.0 and <6.0 signals/cell		
Negative	<4.0 signals/cell	< 2.0 with an average HER-2 copy number <4.0 signals/cell		

HER-2-Human epidermal growth factor receptor; CEP17-Chromosome Enumeration Probe 17

interpreted by two pathologists who were blinded to the other results.

The research slides were processed by the standard protocol of PathVysion HER-2/neu DNA Probe kit for HER-2 study, Vysis LSI EWSR1 (22q12) Dual Color Break Apart Rearrangement Probe for EWSR1 study, and Vysis ALK Break Apart FISH Probe Kit for ALK study. All cases were reported by two pathologists. One interpreted by the fluorescence microscope (Olympus BX61), the other one performed analysis by digital photos created by Applied Spectral Imaging (ASI) model FPCPT0001 camera and GenASIs version 7.2.6.22135 software. The photos that were taken from FISH slides were examined at 100x magnification of at least 4 fields per sample (hot spot). Subjective scores were analyzed without any knowledge of the prior observer results.

Criteria for interpretation

Human epidermal growth factor receptor 2 (HER-2) HER-2 is detected in breast cancer tissues. To ensure the accuracy and efficiency of the HER-2 study results, this research focused on 5 dimensions, which were 1) signal of HER-2, 2) signal of chromosome enumeration probe 17 (CEP17), 3) ratio of HER-2 per CEP17 (HER-2/CEP17 ratio), 4) average HER-2 copy number, and 5) interpretation of the results. 30 cases of HER-2 were tested using both TM and DI methods. The criteria for result interpretation are shown in Table 1.[3]

Ewing sarcoma breakpoint region 1 (EWSR1) gene For EWSR1 gene, 4 dimensions were compared with both TM and DI scoring methods. These dimensions were 1) break apart, 2) normal, 3) percentage of breaking apart, and 4) results interpretation. The result of EWSR1 must be counted at a minimum of 100 non-overlapping nuclei, and it was considered positive when there were >15% nuclei with split signals.[4]

Table 2 Criteria for interpreting ALK[5]

Result	Criteria
Negative	<5 cells out of 50 (< 5/50 or <10%) are positive
Positive	>25 cells out of 50 (>25/50 or > 50%) are positive
Equivocal	5 to 25 cells (10 to 50%) are positive

Remark: If the sample is equivocal, a second reader should evaluate the slide.

- The first and second cell count readings are added and become average percent of positive cells (from 100 cells).
- If the average percent of positive cells is <15% (<15/100), the sample is considered negative.
- If the average percent of positive cells is ≥15% (≥15/100), the sample is considered positive.

Anaplastic lymphoma kinase (ALK) gene ALK gene is detected in adenocarcinoma of the lung tissue samples. The criteria for interpreting ALK results comprised negative, positive, and equivocal measurement, as presented in Table 2.[5] Like EWSR1, ALK cases also focused on 4 dimensions and were compared with both direct visualization and image capture methods. These dimensions were 1) break apart, 2) normal, 3) percentage of breaking apart, and 4) results interpretation.

Statistical analysis

The minimum sample size required for this study, for a confidence level of 95%, was 29.[6] The actual sample size was 40 cases (taken from a total of 400 cases filed in the IOP in 2016). Each entity or case was examined twice to obtain a pair of observations, which were compared using paired *t*-test (two-tailed).[7]

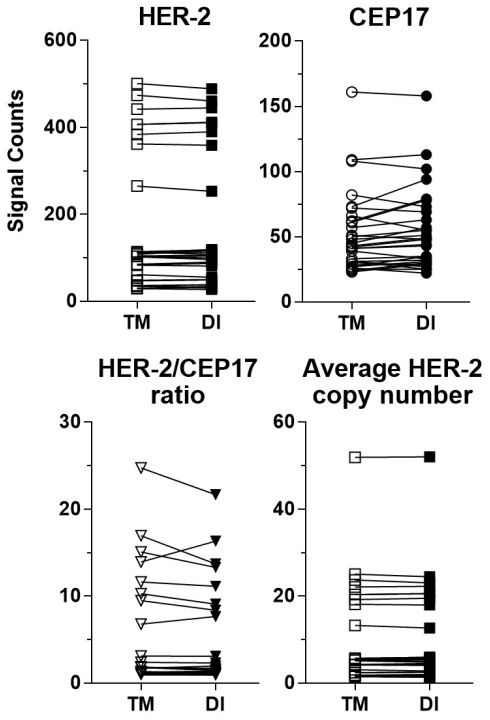

Figure 1 HER-2 results, comparing HER-2 and CEP17 signals, HER-2/CEP17 ratio, and average HER-2 copy number, between those obtained from traditional microscopy *vs* digital image, from 30 cases, 20 cells/case. Each data point represents one case; lines connect data from the same case. No statistical significance was found in the differences between values obtained from either method ($p < 0.05$, paired *t*-test, two-tailed). Abbreviations: CEP17, chromosome enumeration probe 17; DI, digital image; HER-2, human epidermal growth factor receptor 2; TM, traditional microscopy.

Results

HER-2

HER-2 and CEP17 FISH signals were counted in 30 cases for 20 cells/case. The blinded results of TM and DI display are shown in Figure 1 and Table 3. There were no significance differences between TM and DI results of HER-2 and CEP17 signals, HER-2/CEP17 ratio, and average HER-2 copy number (paired *t*-test, two-tailed; p = 0.853, 0.086, 0.123, and 0.691, respectively; Figure 1). Result interpretation was in total concordance (Table 3).

EWSR1

These results were obtained by counting EWSR1 FISH normal and break apart signals of 5 cases for 100 cells/case. The blinded results of TM and DI display are compared in Figure 2. There were no significance differences between TM and DI results of break apart, normal, and percentage of breaking apart (paired *t*-test, two-tailed; p = 0.569, 0.552, and 0.569, respectively; Figure 2). Result interpretation of EWSR1 was in total concordance (Figure 2, *inset*).

ALK

The results were similarly acquired in 5 cases by counting ALK FISH normal and break apart signals like in EWSR1 FISH, but in 50 cells/case. The blinded results of TM and DI display are compared in Figure 3. There were no significance differences between TM and DI display results of ALK break apart, normal, and percentage of breaking apart (paired *t*-test, two-tailed; p = 0.108, 0.893, and 0.108, respectively; Figure 3). The concordance of ALK result interpretation was complete (Figure 3, *inset*).

Table 3 HER-2 result interpretation.

HER-2/CEP17 ratio		Average HER-2 copy number		Result interpretation	
TM	DI	TM	DI	TM	DI
2.38	2.31	5.70	5.90	P	P
9.48	8.38	23.70	23.05	P	P
3.11	3.09	25.05	24.45	P	P
11.64	11.14	19.20	19.50	P	P
24.74	21.68	51.95	52.05	P	P
13.92	16.32	18.10	17.95	P	P
16.96	13.70	20.35	20.55	P	P
10.28	9.09	22.10	22.25	P	P
6.79	7.66	13.25	12.65	P	P
15.07	13.29	20.35	20.60	P	P
1.16	1.11	1.80	1.50	N	N
1.33	1.08	1.80	1.90	N	N
1.10	1.18	1.70	1.90	N	N
1.20	1.15	3.05	2.75	N	N
1.15	1.11	2.35	2.45	N	N
1.13	1.24	1.75	1.55	N	N
1.17	1.16	2.40	2.50	N	N
1.00	.93	1.45	1.35	N	N
1.26	1.10	1.45	1.60	N	N
1.20	1.36	1.50	1.36	N	N
1.15	1.17	4.15	4.05	E	E
1.27	1.36	5.20	4.95	E	E
1.79	1.45	5.55	5.65	E	E
1.16	.95	4.25	4.50	E	E
1.87	1.52	4.20	4.35	E	E
1.71	1.96	5.65	5.40	E	E
1.82	1.52	5.55	5.95	E	E
.94	.94	5.10	4.80	E	E
1.82	1.68	5.20	5.30	E	E
.99	1.03	5.40	5.85	E	E

CEP17, chromosome enumeration probe 17; DI, digital image; E, equivocal; HER-2, human epidermal growth factor receptor 2; N, negative; P, positive; TM, traditional microscopy.

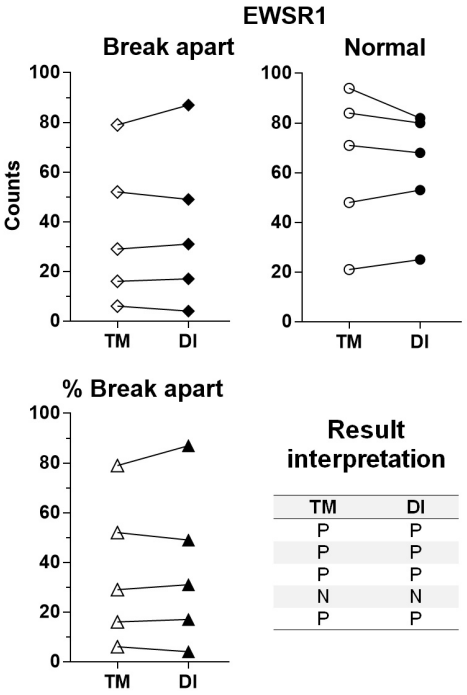

Figure 2 EWSR1 results, comparing EWSR1 break apart, normal and % break apart, between those obtained from traditional microscopy *vs* digital image, from 5 cases, 100 cells/case. Each data point represents one case; lines connect data from the same case. No statistical significance was found in the differences between values obtained from either method (p < 0.05, paired *t*-test, two-tailed). *Inset*: ESWR1 result interpretation. Abbreviations: DI, digital image; ESWR1, Ewing sarcoma breakpoint region 1 gene; N, negative; P, positive; TM, traditional microscopy.

Discussion

Retrospective analytic research has certain advantages which were why this study type was selected. It is possible to conduct such a study on a smaller scale, and it can be completed within a shorter period of time. Retrospective analytic research is also relatively less costly, and with the bulk of the resources focused only on data collection. This kind of studies can accurately and efficiently analyze multiple outcomes and are known for addressing rare diseases.[8] However, there are possible significant biases to be aware of, such as selection or misclassification bias, as well as relying on others' accurate record keeping.

The research results showed that there were no significant differences in all cases (HER-2, EWSR1, and ALK) between TM and DI methods. This is similar to Peters[9] and Hann *et al.*,[10] who examined the

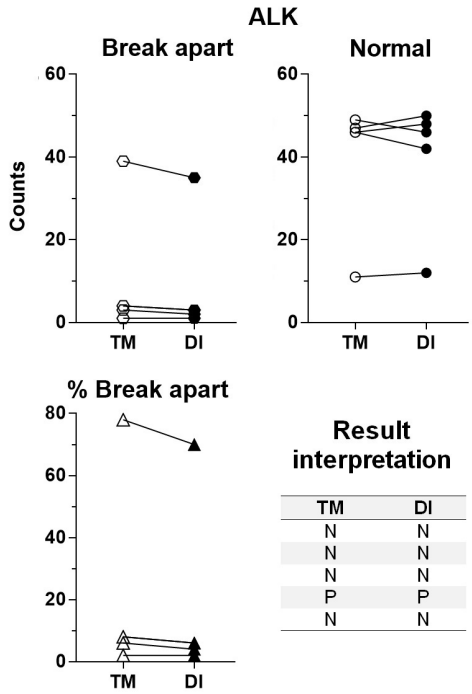

Figure 3 ALK results, comparing ALK break apart, normal and % break apart, between those obtained from traditional microscopy *vs* digital image, from 5 cases, 50 cells/case. Each data point represents one case; lines connect data from the same case. No statistical significance was found in the differences between values obtained from either method ($p < 0.05$, paired *t*-test, two-tailed). *Inset*: ALK result interpretation. Abbreviations: ALK, anaplastic lymphoma kinase gene; DI, digital image; N, negative; P, positive; TM, traditional microscopy.

effectiveness of Image Capture and found that DI could produce images with high resolution for identification of cancerous cells.

Using the TM method can be inconvenient, disturbing pathologist workflow, and time constraining, since the procedure must be carried out at once before the color of the signal fades away. Also, a pathologist must be present during the process in case the technician who scores the signal faces any issues.

Unlike the TM method, the DI method does not have to be carried out immediately. The technician can capture the representative images of selected representative fields using a standard fluorescence microscope with attached camera and scoring signal at any time. Furthermore, a pathologist does not need to be present.

The DI method has several excellent advantages. For example, it can improve subjective interpretation in HER-2 expression,[11] can be used to evaluate the break-apart signal,[12] and has a high concordance rate between the traditional method of FISH analysis and the digital FISH analysis.[13] Other advantages are that the digital diagnostic image and data can be retained for many years following standard retention requirements and the pathologist can send digital images for a second or third opinion. This method is also good for timing and human usage.

Conclusion

This research was blinded and compared the use of traditional microscopy (TM) with digital image capture (DI) method for fluorescence *in situ* hybridization (FISH) analysis in HER-2 (breast cancer), EWSR1 (sarcoma), and ALK (lung cancer) cases. After testing via paired *t*-test, all the cases showed *p* value levels higher than 0.05. Thus, these two methods are equivalent and give the same results.

Because fluorescent signals tend to fade and photobleach with time, we recommend that the Institute of Pathology (IOP), Ministry of Public Health (MPH), Thailand, should consider using the digital image capture (DI) method as the standard practice to improve pathologist and laboratory workflow and reduce medical budget that the Thai government has to spend. Moreover, it is more convenient and effective regarding human usage.

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Conflict of Interest

None to declare.

References

1. Fan YS. Molecular cytogenetics. Protocols and applications. New York: Humana Press; 2003.
2. Jain KK. Textbook of personalized medicine. New York: Springer; 2009.
3. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, *et al*. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update (ASCO–CAP HER2 Test Guideline Recommendations 2013). J Clin Oncol. 2013; 31: 3997-4013.
4. Bridge RS, Rajaram V, Dehner LP, Pfeifer JD, Perry A. Molecular diagnosis of Ewing sarcoma/ primitive neuroectodermal tumor in routinely processed tissue: a comparison of two FISH strategies and RT-PCR in malignant round cell tumors. Mod Pathol. 2006; 19: 1-8.
5. Abbott Molecular oncology and genetics catalog, 2015-2016.
6. Hulley SB, Cummings SR, Browner WS, Grady DG, Newman TB. Designing clinical research. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2013.
7. Hsu H, Lachenbruch PA. Paired t test. In: Wiley encyclopedia of clinical trials. [Published online 19 September 2008] Available from: http://onlinelibrary.wiley.com/doi/10.1002/9780471462422.eoct969.
8. Mann CJ. Observational research methods. Research design II. Emerg Med J. 2003; 20: 54-60.

9. Peters A. Digital image elasto-tomography: Mechanical property reconstruction from surface measured displacement data. New Zealand: University of Canterbury; 2007. [Thesis]
10. Hann CE, Chase JG, Berg C, Brown RG, Elliott RB, Xiao QC. Specialised image capture systems for a DIET breast cancer screening system. Proceedings of the ASME/IEEE International Conference on Mechatronic and Embedded Systems and Applications and the 19th Reliability, Stress Analysis, and Failure Prevention Conference; 2007 Sep 4–7; Las Vegas, Nevada, USA; 2007. p. 169-78. Available from: http://proceedings.asmedigitalcollection.asme.org/proceeding.aspx?articleid=1604442.
11. Skaland I, Ovestad I, Janssen EA, Klos J, Kjellevold KH, Helliesen T, et al. Digital image analysis improves the quality of subjective her-2 expression scoring in breast cancer. Appl Immunohistochem Mol Morphol. 2008; 16: 185-90.
12. Laurent C, Guérin M, François-Xavier F, Thuries V, Jalabert L, Brousset P, et al. Whole-slide imaging is a robust alternative to traditional fluorescent microscopy for fluorescence in situ hybridization imaging using break-apart DNA probes. Hum Pathol. 2013; 44: 1544-55.
13. Liew M, Rowe L, Clement PW, Miles RR, Salama ME. Validation of break-apart and fusion MYC probes using a digital fluorescence in situ hybridization capture and imaging system. J Pathol Inform. 2016; 7: 20. doi: 10.4103/2153-3539.181764. eCollection 2016.